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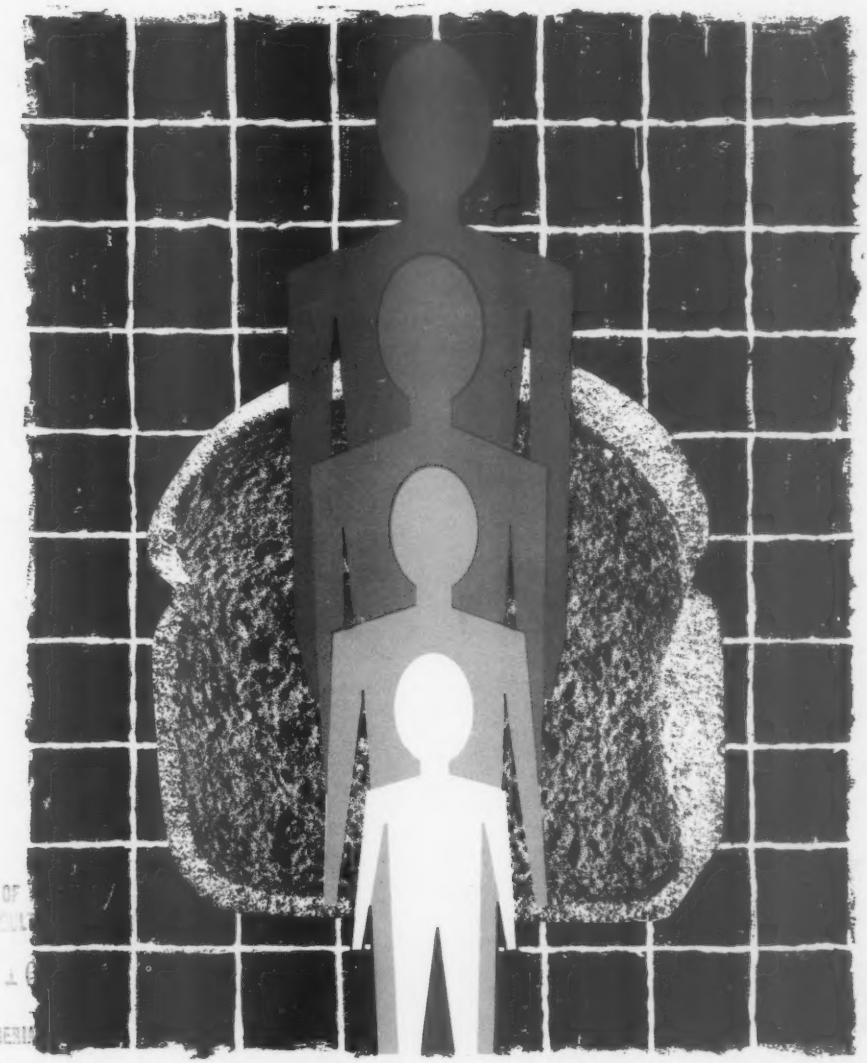

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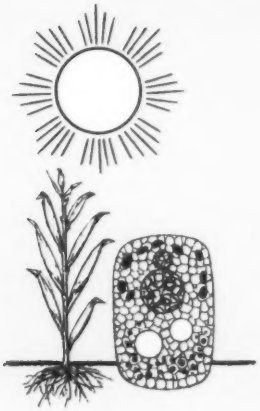
EFFECTS OF NUTRITION ON GROWTH AND PERFORMANCE Page 1



AGRICULTURAL SCIENCE REVIEW

Second and Third Quarter 1970 Vol. 8 No. 2 & 3

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Truth or Opinion

A strange new development is emerging in the world of science: the tendency of both scientists and science writers to go beyond the cold facts of research findings and question what they see or think they see.

The real issue is not whether this practice should be frowned upon. For whether we like it or not, we are living in the "era of the instant replay." No area of human activity can escape the discerning eye, the analytical touch, the rash opinion, the prognosis of happy or somber consequences.

Pondering this situation, the public might well ask: *Is* it pure folly to explore outer space? *Will* we die of starvation if we stop using pesticides? *Are* we wasting our time at the breakfast table eating bowls of cereal? How can we identify truth, biased thinking, erratic beliefs? How can each issue be judged quietly and fairly?

Perhaps, at the moment, we ought to be content that the debate is continuing. As a matter of fact, we might more readily separate truth from opinion if the debate intensified. Both scientists and science writers have a deepening obligation to discard the typical descriptive approach of the purist and apply their critical faculties. I admire the small handful of scientists who helped to start the crusade for a cleaner environment—even though they ran the risk of being labelled "headline hunters." I applaud the efforts of some science writers who have had the audacity to question academic data-keepers.

Yes, the debate ought to continue. And when the time comes for making up our minds, we have no more logical recourse than to turn to a few yardsticks that for centuries have proved to be valid measurements for human actions and utterances: the integrity of the person who makes the judgment, the unrefutable evidence of sound data, and—if we want to wait that long—the finality of historical hindsight.—W.W.K.

NOTE: This issue of Review, which is about twice the size of a normal issue, consists of two numbers: Vol. 8, No. 2 and 3, Second and Third Quarter, 1970.

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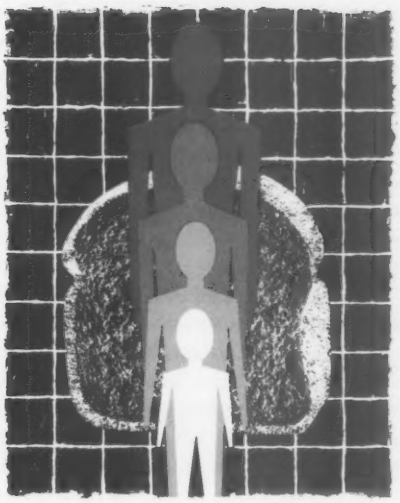
EFFECTS of NUTRITION on GROWTH and PERFORMANCE



H. B. YOUNG

THERE is a growing body of evidence that malnutrition in early childhood may cause stunting in survivors not only in physical growth but possibly also in mental development. Realization of the extent and gravity of this situation, which may produce future generations progressively less able to break out of the vicious circle, has stimulated research workers to delineate the problem and to explore possible solutions.

Prevalence surveys of protein malnutrition in Africa, Central America and Brazil have drawn attention to the magnitude of the problem. During the past two decades, protein-calorie malnutrition has been increasingly recognized as a major factor in growth failure and in childhood morbidity and mortality.

Despite considerable research work, a number of important problems remain unsolved. Among these are the relative contributions of poor nutrition, infection, climate, and cultural factors to growth factors and the permanence of physical and mental growth failure, that is, the extent of the "catch-up" growth phenomenon following intervention at differing ages. In prevalence growth surveys, it is also important to control genetic factors. However, many factors usually attributed to genetics (such as height, body proportion, cranial size, etc.) are also closely related to nutritional status.

Physical Growth

IN satisfactory environmental situations, the growth of children is probably similar in most parts of the world. Failure to reach potential is accounted for by environmental deficiency—in most cases a synergism between nutritional deficiency and infection.

Although this area is still controversial, malnutrition in expectant mothers appears to have surprisingly little effect upon the heights and weights of full-term newborn infants, although there is a relationship between poverty and prematurity in Western societies.

Post World War II studies at Wuppertal, Germany (*16*),[1] showed that after allowance for shortening of gestation, the birth weights of infants during a period of nutritional hardship were reduced by less than 160 grams—about 4 percent—while body length was apparently not affected when gestational length was allowed for. Smith (*22*) reported somewhat greater decreases of birth weight in Holland, again with comparatively less effect of

This article was adapted from a paper presented by the author at th International Conference on Amino Acid Fortification of Protein Foods, Mass. Institute of Technology, Boston, Mass., Sept. 16–18, 1969.

[1] Italic numbers in parentheses refer to Literature Cited p. 7.

hardship upon body length. Both these studies separated out such factors as sex of child and first and later born which might have a bearing upon body measurements. Another study, which did not consider such factors, is that of Antonov (*2*) who described decreases in expected weight of up to 642 grams during the siege of Leningrad. In none of these studies is there adequate information on the nutritional state of the mother throughout pregnancy.

Reports from Lebanon and Jordan (*12*) state, however, that infants who subsequently show growth failure are comparable with American infants at birth. This is further reported by Phaaron *et al.* (*19, 20*) who also have been unable to demonstrate significant differences at birth in body length and weight between the well-off and the very poor. Our data in Tunis, where we have been doing research work for the past four years, show relatively small differences in weight (0.05 kg) and height (1.6 cm) when new born Tunisian infants were compared with Stuart Meredith Standards. No Tunisian social class differences in weight and height at birth were seen in these observations.

The vast majority of Tunisian infants are breast fed, and it has been shown that in areas of the world widely disparate in socio-economic conditions the physical growth of breast-fed infants for the first six months is comparable with that of well-nourished American babies (*17*). After the first six months in countries where there is much malnutrition and infection, there is progressive failure of growth and a maximum decrement of growth velocity between 18 and 24 months of age (*12, 17*).

Such adverse environmental conditions affect all aspects of physical growth and also skeletal development including both the time of appearance of ossification centers and the thickness of cortical bone. West African infants may be advanced in skeletal maturation until the age of one and a half years, after which there is progressive retardation in skeletal maturation.

Permanence of Effects of Malnutrition

THE available evidence in humans indicates that severe malnutrition in early childhood and infancy is likely to have permanent effects upon ultimate stature and general intellectual ability (*10, 11*). Evidence from animal experimentation has tended to support this point of view. The observations of McCance, Widdowson and Kennedy (*13, 25, 26, 27*) as well as others (*21*) indicate that undernutrition in rats during the critical period of early lactation results in permanent stunting of growth. In other animal species, notably the pig, this phenomenon does not seem to occur; following refeeding, resumption of growth takes place and leads almost to normal size and stature (*23*).

The work of Winick with rats has shown that undernutrition at critical periods of growth by cell division leads to permanent relative reduction of cell number (*30*). But once the full quota of cells for a given organ has been achieved, undernutrition leads only to reduction in cell size, and upon refeeding cells return to normal. These relationships seem most critical in central nervous system components where cell division continues until 5 to 6 months of age (*28*). Further observations by Winick (*29*) of the brains of children dying of malnutrition indicate that these concepts may apply to the human infant. Only further observation and experimentation can test whether or not critical period deprivation plays a role in humans equivalent to that found in certain experimental animals.

The period of greatest cell division should correspond to peak growth acceleration at the macro level. Vulnerability should persist, although decreasingly, just as long as acceleration does not approach zero. The acceleration curve, its peak, and width—and therefore vulnerability—may vary from organ to organ.

It is not clear why there is limitation of catch-up growth in certain animals such as the rat. Appetite conditioning does not appear to be a factor as it has been shown that such animals do not consume less. But there is some evidence of poor absorption and also of altered metabolism (*14*). There is also some evidence that injection of growth hormone may practically eliminate limitation of catch-up growth in the rat (*3*).

Biochemical Changes

IT has been reported that undernourished children have creatinine excretions low for chronological age but normal for "height age" (*1*).

In summarizing relationships between nutrition and biochemical processes, Cravioto (*5*) referred to a failure to develop glucose-phosphatase activity

in malnourished pigs. He also reports in malnourished children, a defect in the enzyme system that converts phenylalinine into tyrosine. Cravioto also refers to the relationships between nutrition and body composition; malnourished children have a body composition which corresponds to a less mature situation than their chronological age would suggest. Specific delay in biochemical maturation in malnourished children has been reported by Dean and Whitehead (*6*). Here there were abnormalities in the metabolism of histidine and phenylalinine apparently due to enzyme defects, a situation which was corrected following treatment.

Metcoff *et al.* (*15*) have recently reported extensive studies on 13 patients hospitalized with kwashiorkor. Muscle cell H_2O and Na were increased while intracellular K and metabolites were decreased.

Blood changes have been extensively reported in poorly nourished subjects. Serum proteins and in particular serum albumin and the albumin/globulin ratio are sensitive indicators, and haemoglobin and haematocrit levels are also useful measures. The hydro-oxyproline/creatinine index and plasma amino acids have been extensively used but they are not easy techniques and may not reflect nutritional status as one protein meal may be enough to radically change the levels.

Physical Performance

THERE have been relatively few studies relating status of malnutrition with physical performance. Fernando Viteri at INCAP in Guatemala has done some very interesting work but it does not appear to have been published.[2] Similarly, Dr. Jose Mendez has done work in Japan but this too has not yet been published to my knowledge. In our own experimental work, some physical performance tests in children with differing levels of nutrition showed significant differences between privileged and underprivileged in three of the tests. The privileged and well-nourished were superior in dynamic flexibility, balance, and static strength; in the fourth test—cardiovascular endurance, as measured by the Harvard Step—the differences fell just short of the 5 percent level of significance.

[2] Dr. Viteri subsequently discussed some of this research at the MIT conference.

Catch-up Growth

THE evidence available suggests that if infants are subjected early in life, for a sufficient length of time, to poor nutrition conditions it is unlikely that they will return to their expected growth patterns but will remain with a permanent physical growth deficit. Some research workers claim a relatively complete physical recovery following severe malnutrition, a recovery however not reflected in the children's mental development. This view is supported by Garrow and Pike (*9*) who found formerly severely malnourished children, now rehabilitated, to be comparable with other children of the same socio-economic background. However, these comparable children were living in adverse circumstances and it is possible that much growth potential was lost.

It is clear that insofar as physical growth is concerned, there is still controversy as to the degree of catch-up growth. Even less information is available on mental growth, but what there is points more strongly to severe limiting of catch-up growth.

Confounding Factors

A number of other factors—mortality and morbidity rates, resistance to infection, feeding practices, and emotional deprivation—are likely to be encountered in a given nutrition research study and thus may confound the situation.

Infant and toddler mortality rates are high in developing countries. For example, Malaya has an infant mortality rate of 57/1000 and a toddler (1 to 4 years) mortality rate of 34/1000 as compared to 19 and 1 respectively for Australia (*4*). This mortality is combined with a high morbidity especially for infectious diseases and parasites, and as a result interpretations in nutrition research are made difficult.

There is evidence that children with sub-normal growth associated with diets deficient in protein have reduced tuberculin reactions after BCG vaccinations and that a protein-rich diet may increase the response. Similarly, children with kwashiorkor are unable to form antibodies to typhoid vaccine or diptheria toxoid and here also the antibodies are again formed following adequate increase of protein in the diet.

It is also clear that some infections upset nitrogen balance and may alter vitamin utilization. The contribution of parasites, such as ascaris and hookworm, to malnutrition is less clear but must also be taken into account.

Early weaning in developing countries may make malnutrition and infection more probable. Contrary to what many think, the composition of breast milk

remains remarkably constant up to even more than 18 months of feeding, although it has been shown in India that the quantity may fall off appreciably during the second year (*4*). Breast milk then becomes a protein supplement.

In any nutrition work, attention must be given to the quality and quantity of interaction between infant and mother or mother substitute.

Recently I have had the opportunity to review this field. There is some evidence that emotional deprivation may be associated with physical growth disorders, although there is still some controversy here. Patton and Gardner (*18*) have analyzed six case histories where maternal deprivation appears to be related to growth failure. Widdowson (*24*), in orphanage studies in Germany in 1948, noted a clear effect of absence of love and affection upon children who failed to thrive as expected despite adequate supplementation. Similarly in certain English boarding schools, the rate of physical growth has been shown to be less in term time than in the holidays (*7, 8*).

Further observations are called for to confirm and amplify these findings in well-planned longitudinal studies which may also attend to the question of reversibility and take better note of the many nonmaternal variables.

Experimental Work in North Africa

THERE are conceptual difficulties in determining what may be accepted as a "satisfactory" level of health and growth. In some respects, determining the level for health is easier. The reason is that perinatal, infant and pre-school child mortality rates, when carefully collected and analyzed together with morbidity rates, may give a broad idea of the health deficits of a population or particular strata thereof.

Determining a level for growth is more difficult, because a satisfactory level may be accepted as one which approximates genetic potential. But how is such genetic potential to be determined? For many who believe that the potential growth of children is not very different across the world in at least 90 percent of the population, a comparison between countries may be interesting.

Thus, differences in growth by chronological or biological age between children in technically advanced and materially rich societies and those in developing countries may represent a deficit on whatever human variable is being studied, for example, total body height, cardiovascular endurance, performance on intelligence tests. However, there are good reasons that investigators interested in these problems should look even more carefully at the differences between socio-economic groups *within* a given society. Here, there is more assurance of genetic homogeneity, and doubts about the suitability of instruments and methods are less grave because such instruments and methods may be adapted to one society but not to another.

It is only now that even in the United States data are being collected in a systematic manner on socio-economic group differences in physical and psychological development from birth through adolescence. This work is being undertaken under the aegis of the National Center for Health Statistics. Theoretically, the richer a country and the better organized its social services, the fewer differences should be seen in health and growth between rich and poor. A divergence index of this type might well serve as a guide to the social and economic situation of a country and represent a control on the effect of social investments. However, the information necessary for such an index requires careful sampling techniques, appropriate instruments, rigorous training of field research workers, adequate background information on the basic socio-economic situation of the various strata of the population, assurances of reliability, and appropriate analysis. There are, as yet, few places where all these conditions are met.

In addition to such essential acquisition of knowledge of social condition, it is necessary to define and measure relevant environmental variables and attempt to link these with the ecological situation. The definition of one of the major factors, nutrition, is easy although its measurement presents considerable difficulties of evaluation. The study of the levels of hygiene is simpler; but when one comes to the matter of measuring levels of parental nurturance or levels of use of mass media, the problem becomes much greater. Nevertheless, it is important to acquire experience in such measurements and, after the suggestive associations provided by cross-sectional material, then proceed to an attempt to demonstrate causative relationship by limited longitudinal studies.

To this end, therefore, our team began in 1966 a study in Tunisia of the health, growth, and social

behavior of children. This study involved several step-like procedures related in part to the lack of growth data in the country concerned:

1. To test the working hypothesis that there would be substantial growth and health gaps between the middle classes and the poor within that culture. Our intention in this phase of the study was not to assess the magnitude of such differences, but to find out if such differences really existed and in what direction.

2. Analyze the data from the above study to provide some insight as to the degree of growth and health deficits presumably related to the environment and the association of these with environmental variables—nutrition, family income, father's occupation, and mother's educational level.

3. Identify sensitive areas worthy of further investigation—nutrition and infection in young children and those aspects of socio-economic condition that affect child care.

4. Collect background data to adequately document socio-economic class differences and provide normative growth data.

5. Manipulate key environmental variables in order to upset predictions.

Our preliminary results clearly indicate that much remains to be done to improve precision in methodology. Nevertheless, these results are encouraging enough to warrant a further and longer experiment.[3]

A quantity of information has been collected on the food consumption of the families and there are clear indications as to what further information should be obtained and how this should be done.

There is support for the hypothesis that the high protein supplement is, in fact, associated with increased bone muscle circumference. If the number of children had been larger and the time longer, and, most of all, if the seasonal changes had been observed by a full year's study, it is possible that significant differences would also have been reached in respect to body length.

Of considerable interest is the change for the better in the health index of the high protein supplemented group. It is possible that the differences in the Milestone Index arise, at least in part, from this.

In conclusion it appears that longitudinal studies of children with and without supplementation are feasible and some physical growth variables appear as of potential use. The marked seasonal growth variation in malnourished children suggests that studies should continue for a minimum of one calendar year. It is recommended that prior to commencement of such studies in developing countries, a clear picture should be obtained of socio-economic group differences within the country concerned on the variables whose use may be contemplated.

[3] Many of these preliminary results were presented in the full conference paper on which this article is based. It is suggested that interested readers obtain a copy of the full proceedings of the conference when they are available.

The marked differences, on a wide range of variables, between social groups demand further investigation as to the role of the environment as a complete or partial explanation. The present series of observations has pointed a finger at certain environmental variables such as nutrition and infection. The information being accumulated creates a baseline which makes it possible to predict what mean growth and function might be expected of children in the various socio-economic groups in this society provided the changes in the society are not excessively rapid.

Manipulation of an environmental variable by food supplementation, for example, should demonstrate, by comparison with the above baseline data and with controls in a longitudinal study, the effects of such intervention. All participants would be entitled to medical, curative, and prophylactic care. Comparison of the control groups with the background study should determine the part played by infection as a factor contributory to impairment of physical growth, performance, health, and psychological development.

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Instability in Nutrient Values

A study by the Hawaii Agricultural Experiment Station has revealed that certain processed and prepared foods of the same name do not necessarily have the same nutrient values.

Prepared foods of different brands were purchased from restaurants, cafeterias, bakeries, and drive-ins. Frozen and fresh foods of mixed brands were purchased from grocery stores. Some foods were analyzed for cholesterol and fatty acids only; others for all nutrients. Important differences were noted. For example, the proximate values for three samples of English muffins were similar, but the linoleic concentrations were noticeably higher in two samples—suggesting that vegetable oil was used in these and butter in the third sample. Differences were also noted in tuna fish, depending on the method of preparation used.

Hawaiian food scientists believe that the difference in values could have been influenced by the state of dehydration, types of fats and oils used in cooking, and the individual practices of bakers, food processors, and cooks. They noted that, in the absence of exact specifications, average values of foods need to be used with an awareness that they may obscure wide variations in prepared dishes from different sources.

This study, supported in part by the National Heart Institute, was conducted to evaluate dietary and other ecologic factors in Hawaiian and Japanese men with and without coronary heart disease in an effort to learn what factors best correlated with the high mortality rates from coronary heart disease among Hawaiians.

FOOD PRODUCT DEVELOPMENT RESEARCH

R. G. GARNER AND G. J. MOUNTNEY

FOOD product innovation and new process developments have made the 1960's good years for the American consumer. A survey of the growth in food products for the decade 1957–1966 shows a strong consumer preference for convenient, low calorie and high protein foods. The fact that about two-thirds of the products available to housewives did not exist 15 years ago underscores the importance of innovation in food products to industry growth and consumer satisfaction.

Even more changes in the kinds of food consumed and the forms in which foods will be bought may be expected in the 1970's. Not only will new products and concepts be needed, but also a continued population growth will require greater attention to the maintenance of adequate supplies through reduction of losses at all stages of distribution, storage, and use (*13*).[1]

Coupled with the domestic needs is a growing export market to areas of the world where food supplies are inadequate either because of insufficient cropland or underdeveloped agricultural technologies.

New Era Emerging

BECAUSE of changing consumer habits and the numerous advances in food processing and marketing, a new era of formulated foods is emerging. Natural farm products that have traditionally been regarded as complete foods to be eaten as part of a meal—such as milk, potatoes, and grain—have become sources of raw materials to be utilized as

This article was adapted from a paper presented by the authors at the 67th annual meeting, Association of Southern Agricultural Workers, Memphis, Tenn., February 2, 1970.

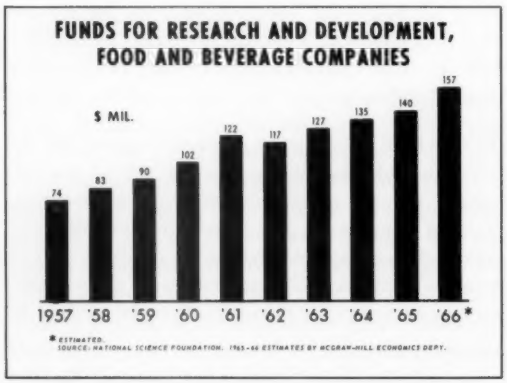
[1] Italic numbers in parentheses refer to Literature Cited p. 52.

building blocks for new foods, especially designed to meet specific, changing needs (*7, 8, 9, 10*).

The new formulated foods include those that are neither synthetic nor imitation. Foods that can be tailored into any desired form or concept such as vegetarian, kosher, polyunsaturated, high or low in carbohydrates or animal or vegetable fat, with or without added vitamins or minerals and precisely controlled calorie content will be a part of the new era. These foods may be precooked, refrigerated, frozen, canned or dried. They may resemble traditional foods or have an entirely novel basic textured structure.

Although traditional agricultural commodities are expected to maintain their position as major sources of raw materials for food, the increased emphasis on processing will intensify both intercommodity competition and competition from non-agricultural products (*5*).

As a guide to food product and process research, it is appropriate to take the broad view that a new food product or process is any product or process that offers the user a new or improved appeal. This viewpoint avoids indulgence with the varying interpretations of whether a product is new and novel or merely improved, since there is no single correct definition of a new product or process that can serve equally well for all purposes. This new or improved appeal may have its origin in many different sources including: utility, variety, convenience, safety, function, cost, flavor, odor, nutritive value, stability or keeping quality, appearance and handling or use advantage. Furthermore, any processed food may be called a convenience food if the processing it has received saves the time of the user or facilitates its marketing or distribution.



The Basic Concepts

THE basic concepts in food product and process development have been described extensively. Hedges (*6*) has emphasized three main stages: idea generation; screening of ideas; and implementation or development. Clausi (2) has pointed out the need to adapt foods to changing consumption trends. A screening guide that identifies technology factors, marketing factors and profitability factors to consider in new product development has been described by Pinkos (*12*). According to Ricker (*14*) the success of a new product will depend on the effectiveness of two marketing requirements: (1) the product concept and (2) the physical characteristics of the product. Diehl (*4*) has advanced the "method approach" as a way to stimulate systematic generation of new product ideas. Basically, this method enables a developer to view a food product as a consumer does, for example, English muffins as an item for breakfast rather than as baked goods.

According to Bird (*1*), the key factors in the succesful development of new foods are that each new product must satisfy a latent demand, be technologically sound and economically feasible, and offer the innovator opportunity to gain by injecting his know-how and his capital into the market situation. To these might be added the reminder that the functions of food are many and varied, but primarily to provide: (1) sustenance for maintaining the body, (2) sensory gratification, (3) a source of emotional security, and (4) a desirable adjunct for social occasions.

It has been estimated that the initial costs of commercially marketing a new food product on a national scale may exceed $1 million (*15*). Because of the high cost and inherent risks in commercializing a new food product, industry must be in position to exploit the advantages of new product introduction. Many techniques have been used to predict the market potential at retail and institutional levels. Perhaps the most critical problem of new product development is to bring, as early as possible, the realities of consumer needs into line with the actualities of materials and processes. The basic task of development is to convert the technological uncertainties in the proposed product venture to a set of

quantified risks. This is the job of industry. Investment in product development can be justified only if these risks, together with the other risks of the marketplace, can be balanced by potential gain.

It is obvious that food product development is the first phase of a goods-market-consumer-oriented sequence whereby an idea is developed, manufacturing is started, a product is distributed and finally, a consumer, through his choice to purchase, votes on the success of the enterprise. However, the economics of progress often require building on a product that already exists, that is, product improvement rather than entirely new ideas.

Food Science Contributions

IT may seem almost presumptuous to discuss what food researchers may contribute to food product development. Yet, food scientists have a vital role to play with regard to idea generation, product concept and origin and basic research on properties of foods, function of constituents, the role of ingredients, and the way ingredients affect properties and cost of a product.

Traditionally, State experiment station research on foods has been related to natural food forms such as milk, meat, and bread. Although food science and technology has enriched the variety of our foods and their availability, there remain critical areas where research and technological developments may further meet the needs of people. The opportunities to strengthen research efforts in food science range all the way from quite fundamental biochemistry to characterization of the properties of agricultural food materials, as well as work with particular commodities to improve their utility, and new product and process development involving new concepts.

The real issue is: Can the food science discipline afford to do otherwise? Can we anticipate and lead and initiate constructively? Can we assist in the fight against hunger? Or will we spend our time fighting rearguard actions, struggling to maintain markets, and protecting our products from synthetics and substitutes? Can food scientists, instead, be creative?

Food is such a fundamental product of farm and factory that agricultural interests cannot afford to adopt anything less than an all-out program of research. For decades, major attention has been given to increasing production in the field and feedlot. Greater attention must now be turned to marketing, to developing new products and by-products, and to more complete utilization of agricultural resources. It will be necessary to find ways to conserve resources, time, talent and agricultural raw materials. Many of the present products are less than perfect and improvements can be expected. New variations and varieties are possible. There is still the challenge of reducing the time in preparation and ease of preparation. Safety and wholesomeness must be protected. The field of special, alternate, or substitute products has not been fully exploited.

Role of Experiment Station

IN a State agricultural experiment station setting, choice of endeavor becomes extremely important. Obviously, a station scientist or his institution cannot exploit the product by marketing it in competition with private industry. The station food scientist can, however, do the work necessary to show the potential of a product, determine the properties of the agricultural commodity—and he can generate product ideas. He can identify basic properties of agricultural commodities. He can plan how to improve present products thereby enhancing their convenience, value, and identity. The scientist must ask: Will this work fit my program of research? Is it likely to be picked up and exploited by industry?

Will it create additional markets for agricultural products? Will it improve present products? Can it meet legal requirements so that it is likely to be developed by industry?

A number of product prototypes developed at State agricultural experiment stations show the potential of innovative research with food. For example, Collins and Ruch (*3*) of the Tennessee Station have developed a high protein snack food by breading and deep-frying vegetable soybeans. Minnesota food researchers have developed three low-fat cheeses that have been well received by consumers. Numerous other examples could be cited.

Efforts directed to improving present products are noteworthy. For example, several important developments have occurred since the first satisfactory sweet potato flakes were produced. It is now possible to process uncured sweet potatoes into flakes by two methods: (1) adding a beta-amylase enzyme to the puree and (2) rapidly heating the puree to 175°F to activate the natural enzymes. Furthermore, great strides have been made in processing the different varieties of sweet potatoes. In addition, several important processes have been modified and improved—for example, speeding up drying rate on the double drum drier. Improved color can be achieved by adding pyrophosphates to the puree. The manner in which processed white potato products have reversed the declining consumption curve is an excellent example of what high quality, concentrated research effort can accomplish.

Even the peanut has yielded to research. Partially defatted peanuts are now well established products and dry-roasted peanuts are familiar to all. Effects on nutritive value and stability of peanut proteins have been investigated (*11*). A product as old as the pickle has yielded to improvement. A major breakthrough has been made in food fermentation with the development of an effective process for pure-culture fermentation, including the pickle.

New Concepts in Food Science

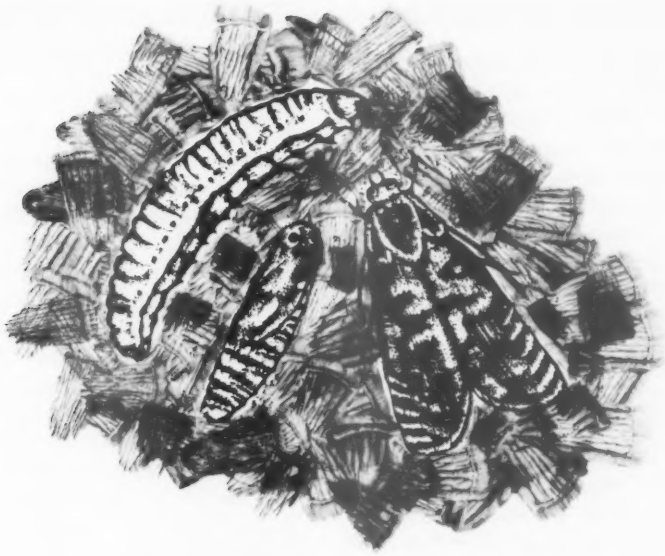
ONE of the prime needs of food science is to develop a data bank of basic research to fully characterize the properties of agricultural food commodities. For example, continuous cheese making systems will likely be perfected in the future as more knowledge is gained of milk protein-casein characteristics, of compounds important in cheese flavor, of mechanisms for developing the flavor, and of the technology of handling curd and milk during the continuous process. Pepsin has been used successfully for milk-clotting in the direct acidification process. Because enzymes affect the body characteristics of cheese, it is possible that use of enzymes combined with various types of acidulants could allow tailor-making of curd and cheeses with well-defined properties.

The concept of agricultural commodities serving as "ingredients" in foods must be further explored and expanded. The same is true for the concept of process modification. If the contribution of each ingredient or processing step is known, the source of product variation may be readily traced and controlled. Many foods are emulsions, and the attaining of emulsion stability is often one of the more vexing problems encountered in the development of food products. Several agricultural products such as meat and egg proteins have functional characteristics useful in modifying the properties of other foods.

Food scientists are beginning to face a totally new problem today that is unique to the 1970's—that of environmental pollution and waste disposal in the food industry. The situation is serious enough that it seems advisable not to make or process a food product without first looking down the road past the consumer and even beyond to the ultimate disposal not only of the waste products that accumulate along the way but also the containers and food packages. More attention must be given to such steps as reduction, re-use, recycling, and incineration. Some food industry byproducts and waste materials might find greater utilitarian value in other segments of the economy; this area invites serious exploration and study.

* * * *

The problems facing the food industry today are as ancient as bread—the first convenience food made by the Egyptians 5,000 years ago—and as new as tomorrow. The only difference, perhaps, between the problems of yesterday and those of today is in the scope and variety. Any measure of success that food science and technology might achieve will rest solely on the quality of talent and other appropriate resources assigned to the task. It seems reasonable to expect that State experiment station researchers can contribute significantly to this important area of research. (*Continued on page 52*)



ENDOCRINOLOGICAL
STRATEGIES IN INSECT CONTROL

HOWARD A. SCHNEIDERMAN, A. KRISHNAKUMARAN, PETER J. BRYANT AND FRANTISEK SEHNAL

WE co-exist with more than 1 million species of arthropods, most of which are benign or beneficial. However, there are some 10,000 species which, if left to their own devices, would prevent us from producing the food and fiber necessary to maintain our present population. Should the world's population decrease during the next 30 years (which it probably will not), rather than double (which it probably will), man would still have to exercise continued vigilance against arthropods, or face world famine and disease. Indeed, even with our present efforts at food production, more than half of our fellow men are hungry most of the time. It seems likely that we shall ultimately have to regulate world population size. But, whatever size that is, unless we control arthropod pests, we shall have mass starvation.

Some methods of controlling insects and mites, such as the use of conventional insecticides, are well established in world economy, but are coming under increasing assault because of their ecological side-effects. New approaches are clearly needed. Some novel approaches, such as the use of pheromones as baits, have not yet been exploited extensively, but hold promise. The burden of our argument is that insect hormone analogs may provide effective and specific insect and mite control which would cause little damage to other animals or to plants. It is our belief that these techniques are worthy of the serious attention of applied entomologists.

This article was adapted from a paper presented by the senior author at a symposium on "Potentials in Crop Production," held at the dedication of the Entomology-Plant Pathology Laboratory, New York Agricultural Experiment Station, Geneva, N.Y., May 20, 1969. The original paper, which covered genetic strategies as well as endocrinological, was published in the official proceedings of the symposium.

PHYSIOLOGICAL APPROACHES

EVOLUTION has set two boundary conditions which govern the strategy of insect control. First, the historical fact of a common evolutionary ancestry for all living organisms means that the basic biochemistry of all organisms is the same. They probably have the same genetic code, which appeared about 2 billion years ago. All eucaryotic organisms share the same basic ultrastructure and have membrane-enclosed organelles which probably appeared 1 billion years ago. This means that any insecticides which affect basic metabolic processes and damage specific cell structures will affect organisms other than insects. Secondly, the process of evolution by natural selection favors the appearance, eventually, of organisms resistant to any insecticide. There is no way to bypass these boundary conditions, but recognizing them can help us plan our strategy.

Since both trilobites and protochordates are found in Cambrian rocks, arthropods and vertebrates must have diverged from a common ancestry at least 600 million years ago. During the 600 million years in which these two major groups have undergone divergent evolution, there have been numerous opportunities for biochemical innovation. Many processes such as protein and nucleic acid syntheses, mechanisms of cellular respiration, etc. have not changed. Others, for example the mechanisms of gas exchange, and the hormonal control of reproduction are very different in the two groups. One process peculiar to arthropods is cuticle synthesis and molting. Interfering with the molting process is, *a priori*, an approach to arthropod control. However, since the control of molting seems to be similar in all arthropods, agents which interfere with molting will probably affect all insects, and not be group specific. For group specificity one must exploit processes of more recent evolutionary origin. Sex attractant pheromones are a good example—and these show high specificity. Absolute specificity can be obtained by using various genetic methods.

PHEROMONES

THE study of pheromones—chemicals secreted by insects which influence the behavior of other members of the same species—has made rapid progress during the past decade and has culminated in the identification of a number of these substances including sex attractants and aggregating substances. A typical example is gyptol, a 10-acetoxy-cis-7-hexadecen-1-ol. A few hundred molecules of this compound per milliliter will attract male gypsy moths, but no other species that have been tested are attracted to it. Like all sex attractants of Lepidoptera thus far uncovered, it is an acetate of a long-chain unsaturated alcohol (*15*).[1] Pheromones like gyptol can be used to attract males to baits where they are either killed or treated in some way and released into the population. Treatment may involve genetic sterilization or they may be exposed either to chemicals which produce sterility in females they will ultimately mate with, or to lethal viruses which they will introduce into the population.

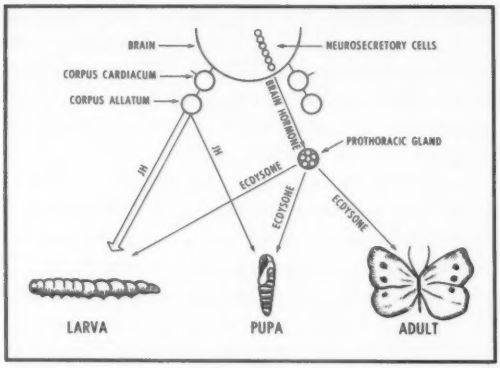
Another technique which appears promising is to permeate the atmosphere with the pheromones so that the males may be unable to locate the females. Alternatively, the natural pheromones may be masked by various agents, notably the long-chain alcohols from which the pheromone was derived. Another use of attractants is to provide an early-warning detection system for various pests so that infestations may be detected and contained before they spread. Ultimately, by judicious use of pheromones we may be able to regulate populations of insects on a regional or even national basis. Indeed for some insects, this appears to be both technically and economically feasible.

HORMONES

IN 1956, Williams (*54*) speculated that insect hormones might provide the basis for a new generation of insecticides. A number of events in the past 3 years have made it possible to test the feasibility of this suggestion, namely: the identification and synthesis of members of 2 of the 3 major types of hormones which control insect development, and the discovery of numerous natural and synthetic hormone analogs, some of which have great group-specificity. We have also learned when hormones act in the life of insects so that we can predict the best possible time to apply them.

Let us begin an analysis of hormone-based insecticides by briefly examining the three groups of hormones that control molting, metamorphosis, and sexual maturation in insects: the brain hormones,

[1] Italic numbers in parentheses refer to Literature Cited p. 24.



An insect's metamorphosis is governed by the interaction of juvenile hormone (JH) and ecdysone. Control of insect populations may be possible by means of synthesized JH or ecdysone, or both, to prevent complete metamorphosis. Ecdysone was synthesized by Syntex in 1966. Certain nerve cells in the brain secrete a hormone which controls the secretion of the sterol hormone, ecdysone. The latter promotes growth when juvenile hormone is simultaneously present. When juvenile hormone is absent, ecdysone promotes the metamorphosis of the larva to pupa to adult. (Courtesy: Zoecon Corp.)

the molting hormones or ecdysones, and the juvenile hormones. The brain hormone, secreted by neurosecretory cells in the protocerebrum, activates the prothoracic glands. These glands in turn secrete one or several closely related steroids, the ecdysones, which cause the insect to molt. The kind of cuticle secreted by the epidermal cells at each molt is affected by a third group of hormones, the juvenile hormones, which are secreted by the corpora allata. In the presence of the juvenile hormones, larvae molt into larvae. However, in their absence, in hemimetabolous insects, larvae metamorphose into adults; in holometabolous insects, larvae molt into pupae and then into adults. Molting is controlled by regulating the release of brain hormone. Maturation and metamorphosis are controlled by regulating the release of juvenile hormone. In simple terms, the ecdysones stimulate the synthetic activities necessary for molting, whereas the juvenile hormones influence the kinds of synthetic activities that occur.

Let us consider briefly the chemical nature of these hormones, their physiological effects and ways in which hormonally active materials may be employed as agents of insect control.

Brain Hormones

A number of hormones are secreted by the insect brain. For example, bursicon brings about cuticular tanning in flies; another hormone appears to induce proteolytic activity in the gut, and a hormone appears to activate the corpora allata in silkworm pupae. The brain hormone with which we are concerned activates the prothoracic glands. To bioassay for this brain hormone, the agent to be tested is injected into a debrained pupa. If the insect molts, then the agent has brain hormone activity or ecdysone activity. The agent is again tested on an isolated abdomen which, of course, has no prothoracic glands. If the isolated abdomen fails to respond, then the agent has brain hormone activity.

Unfortunately, agents other than true brain hormones also activate the prothoracic glands. These include juvenile hormone (*9*), juvenile hormone analogs and various steroids (*39*), injury (*27*), etc. Apparently the prothoracic glands are a poised target organ which can be triggered to secrete by a variety of stimuli. The nonspecificity of the assay for brain hormone led to the initial identification of the brain hormone by Kobayashi and his co-workers as cholesterol (*20*), but this unlikely conclusion has since been changed.

Within the past several years, the laboratories of Ishizaki, Kobayashi, and of Williams have reported the isolation of a water soluble material from isolated insect brains which has potent brain hormone activity and appears to be the true brain hormone (*14, 19, 56*). In the most recent analysis of the matter Kobayashi and Yamazaki present convincing evidence (*59*) that the brain hormone is a protein or polypeptide with a molecular weight of about 20,000. The purest preparations contained about 10 to 15 percent carbohydrate and the substance may by a glycoprotein. About 20 nanograms will activate the prothoracic glands of a brainless silkworm pupa. So far as we know, the prothoracic glands are the only direct target of this hormone, but there have not been enough experiments with purified brain hormone to prove this.

Ecdysone

ECDYSONE causes molting in all insects upon which it has been tested. To bioassay for ecdysone, an isolated larval or pupal abdomen is injected with the extract to be tested. If the abdomen molts or

forms a puparium then the extract has ecdysone activity. Recently it has been demonstrated that simply dipping the abdomens of larval insects into a methanolic solution containing ecdysone is adequate to initiate molting (*36*). In some cases ecdysone may be fed to larvae in the last larval instar where it will stimulate precocious pupation.

Karlson and Butenandt began work on ecdysone 27 years ago. They crystallized it 15 years ago and its structure was announced 3 years ago (*13*). From an analysis of 250 mg. of crystals isolated from 1,000 kilograms of pupae of *Bombyx mori*, Karlson and his associates found ecdysone to be a steroid with the empirical formula $C_{27}H_{44}O_6$ (2_β, 3_β, 14_α, 22R, 25 pentahydroxy-Δ^7-5_β-cholesten-6-one). It causes molting in all insects upon which it has been tested and is the first steroid hormone identified in an invertebrate. In addition to a major component, called simply ecdysone or alpha ecdysone, insect extracts also contain smaller quantities of two other components: ecdysterone (=beta ecdysone, 20 hydroxecdysone, crustecdysone) (*12*) and 20,26 dihydroxyecdysone (*53*). About 3 years ago, Horn and his colleagues in Australia (*10*) isolated ecdysterone from a ton of shrimp. Soon thereafter in 1966, teams of American, German, and Swiss chemists almost simultaneously achieved the synthesis of alpha ecdysone, a synthesis of extreme difficulty since the molecule possesses 10 asymmetric centers (*11*, *18*, *46*). The substance was thus available for extensive testing on insects and other organisms.

The fact that ecdysone is a steroid holds considerable interest, for most insects cannot make sterols. Since the early experiments of Bloch and Rittenberg (*1*), it has been repeatedly demonstrated that labelled acetate is incorporated into cholesterol by tissues of most higher animals via a pathway involving the synthesis of mevalonate and squalene. However, there is no evidence that insects can synthesize sterols from acetate, mevalonate or any other nonsterolic compounds that have been tested. Indeed, all insects that have been studied appear to have an obligatory dietary requirement for sterols. Since ecdysone is a steroid, then either it must be made by modifying a dietary sterol, or new pathways of sterol biosynthesis exist in insects and differ from those reported in microorganisms and in mammals. There is some evidence that ecdysone can be made from cholesterol. Karlson and Hoffmeister (*17*) injected tritiated cholesterol into fly larvae and extracted crude ecdysone. This material was radioactive, and cocrystalization with unlabelled ecdysone yielded pure ecdysone with constant specific activity. They concluded that cholesterol was a precursor of ecdysone.

Insects use the steroids, alpha and beta ecdysone, as molting hormones. However, the situation in other arthropods is less clear. Although beta ecdysone had been isolated from Crustacea by the spring of 1968, no one had demonstrated that ecdysones, or any other chemical agents, could cause molting in any arthropods other than insects. About a year ago we (*22*) began to examine the effects of ecdysone on various arthropods, and we discovered that ecdysones, either injected or applied topically, caused molting in all those tested.

The ecdysone that we employed in most experiments was ecdysterone (beta ecdysone), a steroid which has been isolated from both crustaceans and insects. As experimental animals we selected representatives of the major arthropod subphyla and classes: *Limulus polyphemus,* the horseshoe crab, Class *Merostomata; Araneus cornutus,* a spider, and *Dugesiella hentzi,* a tarantula spider, Class *Arachnida; Armadillidium vulgare,* a terresterial isopod, *Procambarus sp.,* a freshwater crayfish, and *Uca pugilator,* a fiddler crab, Class *Crustacea.* The animals were maintained under conditions in which spontaneous molting was minimal. They were usually divided into three groups: experimental animals which received an injection of hormone in an appropriate Ringer or 10 percent ethanol; injected controls which received only the solvent; and uninjected controls. The effects were unambiguous; injection of 3 to 50 micrograms of ecdysterone per gram live weight stimulated molting in all species of arthropods examined. In many groups of animals, molting occurred almost synchronously. The response was proportional to the dose: the larger the dose, the sooner the molt, and the higher the percentage of animals that molted. Similar results could be obtained by injecting other ecdysones (inokosterone, alpha ecdysone, ponasterone A, and cyasterone) and by topical application of a methanolic solution of ecdysterone.

To test whether the effects were due to some nonspecific action of steroids, a number of other steroids were tested including several synthetic analogs of ecdysone generously provided by Robbins and his

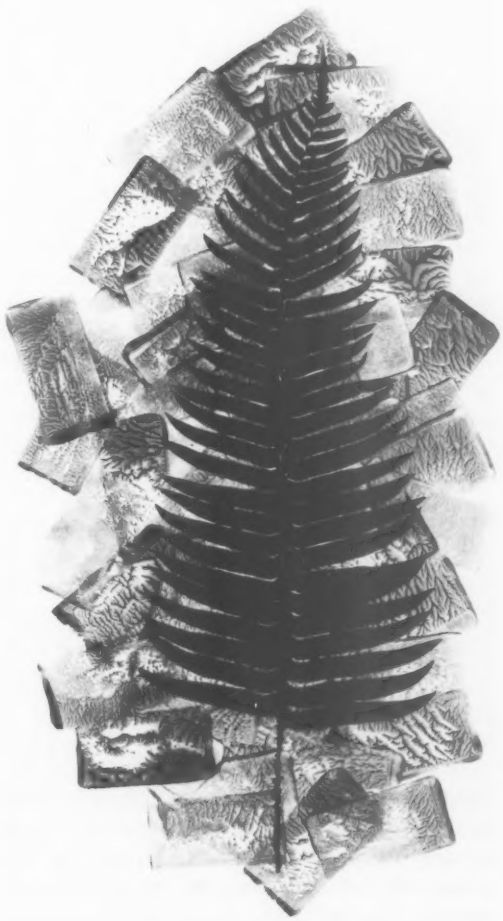
colleagues (*34*). The following compounds, which are inactive in insects, were also inactive in the other arthropods: cholesterol, β sitosterol, α-SEA–1 (Δ^7-5-cholestene-2,3, 14-triol-6-one), β-SEA–4 (Δ^7-5-cholestene-2, 3-diol-6-one) and β-SEA–12 (Δ^7-5-sitostene-2,3, 14-triol-6-one). One compound, β-SEA–1 (Δ^7-5,-cholestene-2,3, 14-triol-6-one), which is slightly active in insects was also slightly active in crayfish. These results indicate that the effects of the ecdysones on arthropods other than insects are specific and are not due to some pharmacological effect of steroids in general (*22*, *23*, *24*).

From these data it appears likely that ecdysones are used as molting hormones by all modern arthropods, and probably were used by the common ancestor of the major arthropod groups in the late pre-Cambrian Period, at least 200 million years before vertebrates evolved. It is possible that this particular chemical control of molting evolved simultaneously with the development of a chitinous exoskeleton, and that it may exist in some non-arthropod groups. Efforts are now underway to examine the effects of ecdysones on nematodes, onychophorans and priapulids—groups which molt and some of which synthesize chitin.

Phytoecdysones—During the past 2 years the surprising discovery has been made that certain plants, particularly evergreens and ferns, contain impressive amounts of substances resembling or identical to ecdysone (*30, 52*). For example, ecdysterone constitutes more than 1 percent of the dry weight of rhizomes of the common fern *Polypodium vulgare*. Thus far, more than 25 phytoecdysones, including authentic alpha and beta ecdysone, have been isolated and characterized, and their structures are all rather similar. A massive effort is now underway in Japan to isolate and identify more of these materials. More than 1,000 species of plants have been examined for ecdysone activity and 40, including ferns, conifers, and flowering plants, have provided active extracts. The occurrence of these phytoecdysones provides a wide range of ecdysones for study.

The question naturally arises why plants synthesize and accumulate exotic sterols like phytoecdysones. But since both ecdysones and phytoecdysones are toxic to insects, it appears possible that plants make use of these agents to protect themselves from attack by insects and perhaps also by nematodes. In this connection it is important to recall that ferns and conifers, unlike flowering plants, do not depend on insects for pollination. Perhaps because they have no need for insects, they have evolved these unusual methods of self defense against insect predation.

Role of Ecdysone—In all arthropods, ecdysone has a profound effect on epidermal cells and causes molting, that is, the separation of epidermal cells from the old cuticle (apolysis), secretion of a new cuticle and ecdysis. It acts in nanogram amounts on intact insects or isolated fragments of insects. In addition to stimulating molting in the absence of juvenile hormone, ecdysone affects imaginal discs, the nervous system and perhaps other tissues, and



causes metamorphosis. Normally, ecdysone is secreted cyclically by the prothoracic glands in response to stimulation by brain hormone. However, epidermal cells are sensitive to ecdysone at all stages in insect development, except in some cases in the adult stage. The sensitivity varies somewhat and cells are much more sensitive at certain stages in the cell cycle (Sehnal, unpublished; Granger, unpublished). However, if enough ecdysone is applied, it will cause insects to molt at virtually any stage. As we shall see below, this is not true of juvenile hormone which acts only at certain stages in the cell cycle.

Toxic Effects of Ecdysone—During a normal molt cycle, the prothoracic glands release ecdysone at a precise rate, and normal development ensues. If insects are experimentally exposed to large amounts of ecdysones or exposed to ecdysones at an abnormal time, it is usually toxic. For some reason, alpha ecdysone appears to be less toxic than the other ecdysones. The reasons for this toxicity are several. In the case of the larvae, ecdysones interfere with wax deposition. If a larva is treated with ecdysones soon after a molt, it often fails to secrete wax, presumably because the insect starts to make a new cuticle before the old one is completed (Krishnakumaran *et al.*, unpublished). Ecdysones are also toxic to larvae about to metamorphose, and to pupae where they may cause juvenile hormone-like effects. Thus, pupae treated with large amounts of ecdysones may molt into non-viable second pupae or into non-viable intermediates between pupae and adults (*21, 57*). For example, if a pupa of *Tenebrio* is injected with 50 μg of ecdysterone, it molts into a second pupa instead of into an adult (Krishnakumaran *et al.*, unpublished).

The reasons for this paradoxical juvenilizing effect were made clear by a number of recent experiments. An autoradiographic study of *Tenebrio* pupae injected with tritiated thymidine revealed that the epidermal cells of normal pupae engage in extensive DNA synthesis before molting, whereas the epidermal cells of pupae given large doses of ecdysone engage in almost no DNA synthesis before molting (Krishnakumaran *et al.*, unpublished). These data suggest that the large does of ecdysone stimulates the epidermal cells to secrete a new cuticle before the cells had time to "reprogram" for adult syntheses. Normally, low concentrations of ecdysone stimulate DNA synthesis and cell division, whereas larger amounts stimulate the immediate synthesis of a new cuticle. Apparently, reprogramming of epidermal cells requires DNA replication. A cell that is making larval or pupal cuticle must "clean its genes" before it can make pupal or adult cuticle (*25, 37, 38*). This reprogramming apparently occurs only at times of cell division. If epidermal cells are forced by a high concentration of ecdysone to make a new cuticle before they have divided, they will continue to make the same old kind of cuticle. These observations have a bearing on the time of action of juvenile hormone and the practical application of hormonally-based insecticides and will be considered again shortly.

A third toxic effect of ecdysone is produced by injecting it into adults. Although this hormone rarely causes molting of adults even when injected in huge amounts such as 3,000 micrograms per gram (*32;* Madhavan, unpublished observations), Robbins and his colleagues have shown that in some insects it causes decreased fertility (*58*). Although the cause of this decreased fertility is not known, the result has the important consequence of extending the effectiveness of ecdysone to adult insects.

Juvenile Hormone

GROWTH in higher vertabrates and higher insects is associated with the non-reproductive, juvenile stage and in both groups maturation is under hormonal control. However, the control mechanisms are wholly different. Maturation in man and other higher vertebrates is promoted by the secretion of maturation hormones, gonadotropins, by the pituitary gland. The juvenile condition in man hinges upon the absence of these maturing hormones. In insects the juvenile condition depends upon the presence of the juvenile hormone, which acts upon the cells themselves, and prevents them from maturing.

To assay for juvenile hormone, the material to be tested is injected into a pupa. If the pupal epidermal cells secrete a second pupal cuticle instead of an adult cuticle, then the injected material has juvenile hormone activity. A topical assay in which an acetone solution of hormone is applied to the insect's skin can also be employed.

From the viewpoint of developmental biology, as well as applied entomology, the juvenile hormone is a remarkable control substance and for the past 11

years with our colleagues Andre S. Meyer and Lawrence I. Gilbert, we have been engaged in isolating it, identifying it, synthesizing various agents that copy its action, and examining its mode of action. Our efforts to isolate and identify the juvenile hormone culminated in rearing a half million Cynthia moths in Japan and the collecting of more than 70,000 Cecropia moths in the United States. In 1965, after 7 years of work and some 40,000 bioassays, we isolated juvenile hormone in pure form and confirmed its purity by a gas liquid chromatographic analysis. From molecular distallation data and gas chromatography, we concluded that its minimum molecular weight was about 300. Also, there appeared to be two active compounds, each of which had about 300,000 times the activity of the material that we started with (*28*).

We hoped to be fortunate enough to identify the juvenile hormone. However, the privilege of deducing its structure fell to Roller and three of his associates at the University of Wisconsin, who, in a decisive piece of work, deciphered the structure of the hormone in 1967 (*35*). It has a molecular weight of 296 and the empirical formula $C_{18}H_{36}O_3$. It is an epoxide of a homosesquiterpenoic methyl ester (methyl 10,11-epoxy-7-ethyl-3,11-dimethyl-2-, 6-tridecadienoate). The correct stereochemistry (2-*trans*, 6-*trans* and 10-*cis*) was established by synthesis (*7, 8, 35*). Since then, a number of most ingenious stereoselective syntheses of this interesting molecule have been achieved (*5*).

In 1968, Andre Meyer in our laboratory completed the identification of the two active compounds we had isolated (*29*). He showed that one compound was identical with the juvenile hormone isolated by the Wisconsin group. He also deduced the structure of the second juvenile hormone we had isolated earlier and showed it to be a lower homolog of the first, differing from it in having a methyl (rather than an ethyl) group at C_7. The new hormone is responsible for about 13 to 20 percent of the endocrine activity in juvenile hormone extracted from Cecropia. Its structure has been confirmed by a stereoselective total synthesis (*16*).

Both hormones are extremely active at the nanogram per-gram level on Lepidoptera, Hemiptera, Coleoptera, and Orthoptera. Thus 0.2 nanograms will cause retention of some pupal characters in 40 percent of *Tenebrio* pupae, 13 nanograms per gram will block metamorphosis in a *Polyphemus* pupa, and 150 nanograms causes extra larval molts in *Galleria*. Whether certain groups of insects have juvenile hormones other than the two described by Roller and by Meyer remains to be proven, but appears likely.

Juvenile Hormone Analogs and Mimics

DURING the past 10 years, several dozen compounds with juvenile hormone activity have been detected in diverse animals, microorganisms, yeasts, and plants (*40, 41*). A few of these have been isolated and identified—notably, farnesol from the feces of the meal worm, *Tenebrio*, by Schmialek (*42*). Another was the well-known "paper factor" of Slama and Williams (*47, 48*) found in paper products derived from balsam fir, which was identified by Bowers and his colleagues (*2*) as the methyl ester of todomatuic acid and called "juvabione."

The list of juvenile hormone mimics has been growing rapidly and includes dozens of terpenoids and their derivatives such as the methyl ester, diethylamine, and dihydrodichloro derivatives of farnesenic acid, natural compounds like phytol, ethers of straight chain alcohols like dodecylmethyl ether. Until recently all of the active compounds which had been discovered bore some structural similarity to authentic juvenile hormones, and we had hopes of comprehending the relation of chemical structure to hormonal activity. This prospect was made somewhat more remote by the recent report of Bowers (*3*) that a number of insecticide synergists such as pyrethrins and carbamates, which are commonly employed to increase the toxicity of insecticides, not only enhance the effectiveness of juvenile hormone analogs, but by themselves have substantial and unmistakable juvenile hormone activity. These synergists include a number of molecules like sesoxane and piperonyl butoxide which are not sesquiterpenoids. The diversity of these compounds requires a reexamination of the relation of chemical structure to hormonal activity. It appears that the action of juvenile hormone in insects can be mimicked by a wide variety of molecules which do not have any obvious similarity except perhaps their size or certain physical characteristics. Also, the fact that these compounds appear to be general synergists and enhance the activity of both conventional insecticides and juvenile hormone analogs should en-

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courage a close examination of their mode of action.

Most of the juvenile hormone analogs and mimics such as dodecyl methyl ether and farnesyl methyl ether affect a wide variety of species. However, some juvenile hormone analogs show remarkable species-specificity. The first of these species-specific compounds to be discovered were juvabione and dehydrojuvabione which were isolated from balsam fir (*2, 4*). These sesquiterpenoids are active at the nanogram level and affect only bugs of the family Pyrrhocoridae (*49*). This remarkable discovery opened up the prospect of isolating or synthesizing juvenile hormone analogs which are highly selective in their action. Recently Slama and his co-workers (*50*) have synthesized a variety of chlorinated derivatives of farnesenic acid esters and shown that they have high stability and species-specificity. By modifying the molecule in various ways they decreased its sensitivity for certain species and increased it for others (*51*). Since juvenile hormone analogs are potential insecticides, the discovery of selective juvenile hormone substances opens the possibility of developing highly selective insecticides, a matter to which we shall return momentarily.

Role of Juvenile Hormone—As mentioned earlier in connection with the bioassay, juvenile hormone acts on epidermal cells and influences the kind of cuticle they secrete. It also affects various internal organs such as the brain, gonads, and mid-gut and prevents their metamorphosis. In some adult insects it has a gonadotropic effect and promotes the deposition of yolk. In Lepidoptera, at least, it activates the prothoracic glands and mimics the brain hormone. In addition to these effects, which may occur during normal life, under experimental conditions juvenile hormone may interfere with embryonic development—a matter to which we shall return when we consider toxic effects of juvenile hormone. It is of some interest that all of these activities are shared by virtually all the compounds that have juvenile hormone activity in metamorphosis tests. Apparently the active sites in the target tissues for all of these juvenile hormone effects are similar.

Cell Cycle—The mode of action of the juvenile hormone is still the subject of much controversy; however, we do have a considerable amount of evidence relating to the timing of hormonal stimuli, and of sensitivity to these stimuli, which give some clues as to the mode of action of the hormone.

For example, in one experiment, *Polyphemus* pupae were injected with juvenile hormone at different times after the initiation of the pupal-adult transformation and simultaneously injected with tritiated thymidine. In the resulting adults, the appearance of pupal patches coincided with patches of cells that showed maximum incorporation of the isotope, a fact which suggests that only those cells which had not completed DNA synthesis had responded to juvenile hormone (Krishnakumaran *et al.*, unpublished).

In another experiment, Sehnal and Novak (*43*) showed that in last stage *Galleria* larvae, juvenile hormone affects only epidermal cells which have not divided, or replicated their DNA. Cells which have divided or replicated their DNA are insensitive to juvenile hormone except at extremely high doses. These and other results indicate that juvenile hormone affects cells only during a limited part of the cell cycle, probably the end of the G_1-period or during the S-period (Sehnal, unpublished). Perhaps it acts on cells before transcription occurs and determines what part of the genome will be used. This idea is now being investigated in several laboratories by DNA/RNA hybridization studies.

Toxic Effects of Juvenile Hormone—The principal cause of juvenile hormone toxicity is the production of intermediates between larva and pupa, pupa and adult, or larva and adult. These invariably fail to reproduce and often fail to undergo normal ecdysis. Such intermediates are not the result of excessive amounts of juvenile hormone. Rather, they occur when cells are exposed to juvenile hormone at abnormal times in their developmental programs. Application of juvenile hormone to larvae at a time when some cells have lost their sensitivity to juvenile hormone, but others have not, leads to the production of intermediates.

A second toxic effect of juvenile hormone is on embryos. Recent experiments (*31, 33, 49*) have shown that when juvenile hormone analogs are administered to adult females or to freshly laid eggs, the embryos fail to develop beyond a stage equivalent to gastrulation, the blastoderm stage. If eggs are treated later in embryonic development, they may give rise to apparently normal larvae which, surprisingly enough, show aberrant development

days or weeks later during the larval stages or at the time of metamorphosis. For example, if juvenile hormone is applied to late embryos, the larvae may hatch and molt several times, but will die before metamorphosis. Juvenile hormone can thus block both embryonic and post-embryonic development if applied at the correct times.

The precise mechanism of juvenile hormone effects in both embryonic and post-embryonic development remains to be elucidated. Juvenile hormone seems to control the reading of genetic information needed for new syntheses (*25, 55*). It seems to permit current genes to operate, but prevents the activation of other groups of genes needed for development. For our present purposes, it is important to note that juvenile hormone affects primarily cells that are synthesizing DNA, a fact which affects the practical application of juvenile hormone analogs in insect control as we shall now see.

HORMONES AS INSECTICIDES

OUR central strategies in developing hormonal methods of insect control are to cause the insect to develop at the wrong season or to interfere with egg production, embryonic development, metamorphosis, or molting. Each of the insect hormones we have discussed can aid us in these strategies.

Promoting Unseasonal Development with Brain Hormone Mimics

THE majority of insect pests are seasonal, emerging in hordes at the time of year when host plants are abundant. How does the insect synchronize its development to fit the agriculturalist's calendar? There are at least two levels of regulators: One is the seasonal changes in temperature, day length, humidity, etc., which are perceived by the insect's sensory systems and regulate the release of the brain hormone. The second level of regulation is the activation of the prothoracic glands by the brain hormone. By judiciously altering the level of brain or prothoracic gland hormones, it should be possible to derange the insect's developmental schedule and induce it to develop at the wrong time.

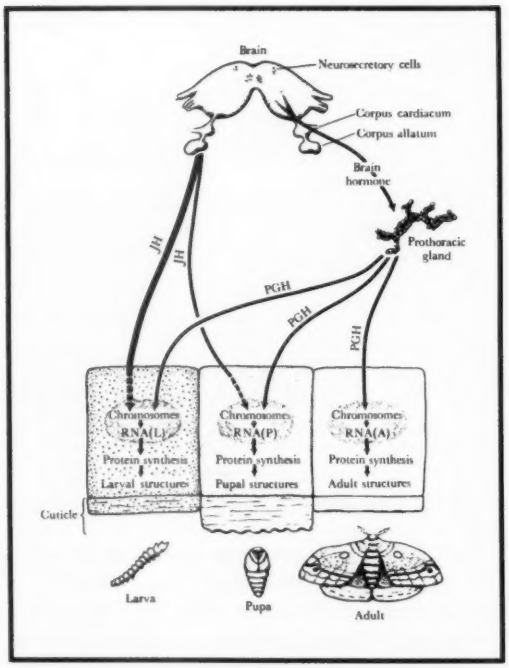
Brain hormone can induce precocious development. If simple methods of applying it could be found, one could break the diapause of various overwintering pests in the fall of the year and cause them to continue development and commit what amounts to "ecological suicide." However, authentic brain hormone is probably a protein and this may make it unsuitable for use as an insecticide. Polypeptides are not stable in nature, cannot easily penetrate the cuticle of the insect, and would be difficult to synthesize economically in large quantities. However, as noted earlier, a variety of substances totally different from authentic brain hormone can activate the prothoracic glands. Perhaps some of these non-protein brain hormone mimics may be useful in causing insects to develop at the wrong season. In this connection it should be pointed out that these agents can be expected to work only on insects which have periods of dormancy and shortly before or during the dormancy.

One non-chemical way to alter indirectly the level of brain hormone is to use photoperiodic schedules which would cause the brain to secrete during the wrong season. It is well established that a single brief flash of light at a certain time before dawn makes some insects behave as if they were exposed to a long day and profoundly modifies the developmental time table. Such treatment may prevent some pest species from entering diapause and may lead to death.

Ecdysones and their Analogs

ECDYSONE and ecdysone analogs hold more promise as potential insecticides than do brain hormone analogs for the following reasons. They affect insects at all stages from newly-hatched larvae to adults. In larval and pupal stages, ecdysones can cause molting at any time in the instar. At low doses (less than 1 microgram per gram) ecdysone will activate the host's own prothoracic glands and initiate precocious molting. At higher doses (1 to 5 micrograms per gram) ecdysones commonly cause death, because the insect produces a defective cuticle: juvenile hormone effects, prevention of wax secretion, etc. In adults, some ecdysone analogs reduce the hatchability of eggs and act as chemosterilants. Ecdysones can be applied topically in various solvents and penetrate the cuticle, and volatile derivatives have been synthesized so that ecdysones can be used as fumigants. Some analogs are effective when fed to insects (*3*). They affect all arthropods and may be equally good as both insecticides and miticides (*22*). Thus far no group-

specific ecdysones have been discovered although some phytoecdysones appear to affect certain groups of insects more than others. Rubrosterone, for example, appears to be especially effective on Diptera (*26*). It is also noteworthy that certain natural phytoecdysones such as cyasterone have been found to be 10 to 25 times as active as authentic alpha ecdysone or ecdysterone, a result which opens up the possibility of synthesizing "super-ecdysones."



Schematic diagram of the principal endocrine organs of the Cecropia silkworm and possible sites of action of their hormones. The larva of this insect molts four (or occasionally five) times. The larval molts appear to be initiated by brain hormone which stimulates the prothoracic glands to secrete the prothoracic gland hormone, ecdysone. At the same time the corpora allata secrete juvenile hormone which favors larval syntheses; then when the larva molts in response to ecdysone it molts into a larva. At the end of larval life the corpora allata cease secreting and thus the mature larva is left with a low concentration of juvenile hormone. At the next molt depending on the time and rate at which ecdysone is released (and possibly also on the amount of juvenile hormone still remaining) the epidermal cells secrete pupal or adult cuticle. Also in response to these hormonal conditions other tissues within the insect either break down or transform into pupal or adult structures. (From Schneiderman and Gilbert)

Another noteworthy feature of the ecdysones is their stability. Also, they are already so widespread in nature as phytoecdysones, that their ecological side effects should be minimal. Their toxicity for mammals appears to be negligible and they do not seem to have androgenic, estrogenic or carcinogenic effects. The only reports of effects on mammals is from workers at Syntex who have discovered that large amounts of alpha ecdysone have some mineralocorticoid effects in mice and cause sodium retention and potassium excretion (*58*).

Recently, evidence has appeared that certain compounds may act as anti-ecdysones and block molting (*3*), a result which opens up a whole new area for insecticide development. Efforts are now underway to synthesize ecdysone derivatives which will be translocated by plants. Also, various conjugated ecdysones are being synthesized in an effort to produce ecdysones which the insect cannot break down. Finally, it has been demonstrated by Robbins and his co-workers that various conventional insecticide synergists like piperonyl butoxide synergize the action of ecdysone analogs.

Thus, all things considered, ecdysones and their analogs look like suitable prospects for agents of both insect and mite control. If early reports that ecdysones act on nematodes are confirmed, then these agents may also prove useful as nematicides. The ecological side effects of the ecdysones will probably prove to be minimal and it appears unlikely that they will be toxic to vertebrates. However, should they prove toxic to annelids (invertebrate animals), then their widespread use might have serious consequences for agriculture.

Juvenile Hormone Analogs as Insecticides

OF all the insect hormones, juvenile hormones seem to have the greatest potential as insecticides. So far as we can predict, they should cause few ecological side effects. They should affect insects during the last larval instar and metamorphosis, by causing the production of intermediates, and during adult life where they act as chemosterilants.

To be effective in producing non-viable intermediates, juvenile hormone must be applied at the proper time and must persist for several days. This matter has been analyzed in some detail (*44*). The main argument is that to produce maximum kill

with minimum dose, juvenile hormone analogs should be applied when some parts of the insect's body are sensitive to juvenile hormone analogs whereas other parts are not. Since juvenile hormone appears to act only at certain times in the cell cycle, these juvenile hormone-sensitive stages can be predicted from studies of the timing of cell division and DNA replication in the integument of developing insects. For example, bugs, locusts, and other hemimetabolic insects usually become insensitive to juvenile hormone a few days after the last larval ecdysis. Consequently, juvenile hormone analogs must be applied shortly after the ecdysis, generally in about the first third of the last instar, or much later, after adult emergence, where they will have an ovicidal effect.

Pupae of holometabolic insects such as Lepidoptera and Coleoptera are sensitive to juvenile hormone only for several hours or at most a few days after the last larval ecdysis. Larvae of most holometabolic insects can only be deranged at the end of the last larval instar. To be effective in the field, juvenile hormone analogs must be applied at the right time and must persist long enough so that all members of the population are exposed to them for an adequate time when they enter their periods of sensitivity to juvenile hormone. Thus, juvenile hormone analogs must persist in the environment for several weeks if they are to be effective.

Potency and Specificity of Juvenile Hormone Analogs

THE available evidence indicates that juvenile hormone-based insecticides may have astonishing potency. For example, Slama and colleagues (*51*) have recently shown that 0.1 nanogram of the methyl ester of trans dihydrochlorofarnesenic acid will cause a *Pyrrhocoris* larva to produce an adultoid which is unable to survive and reproduce. This amounts to 2.5 mg for a metric ton of insects—25,000,000 insects. A dose of only 1 μg per square meter of filter paper was effective (*51*). This means that 10 mg. per hectare could, in principle, prevent the development of *Pyrrhocoris*. Of course, a hectare of soil is very different from a hectare of filter paper and practical problems of application in the field are not yet solved. Another problem which should not be ignored is the fact that many of the potent juvenile hormone analogs are stable chlorinated hydrocarbons which may have ecological side effects similar to those of chlorinated insecticides like DDT. However, the results up to this point are promising and seem to warrant intensive study by applied entomologists.

Experiments have already been completed which demonstrate that topical application of juvenile hormone analogs in laboratory tests can control aphids, coddling moths, lice, locusts, mosquitoes, red cotton bugs, wax moths, and various stored products insects.

Another attractive feature of some of the juvenile hormone analogs is their group-specificity. For example, several juvenile hormone analogs exist which are highly specific for families of insects such as the Pyrrhocoridae which include important pests such as the cotton stainer, *Dysdercus cingulatus* (*50*). These group-specific agents have great interest to the insect physiologist, but from the viewpoint of practical economics, group-specific agents would be useful only if they affected a particularly widespread pest such as the boll weevil or the tsetse fly. (The most successful commercial products have been those with a wide range of activity against a broad spectrum of insects.) Nonetheless they also deserve prompt study by plant protectionists. From a practical viewpoint, it appears likely that the juvenile hormone analogs will be much cheaper to produce than any ecdysone-based insecticide.

A final feature of the juvenile hormone analogs is the novel way in which some of them may be applied. In an impressive experiment, Slama and colleagues (*50*) applied enormous amounts of a juvenile hormone analog to male adults of the bug *Pyrrhocoris apterus* and returned these to a laboratory population. When these males mated with normal females, the females became sterilized by the juvenile hormone analogs. Moreover the females had received so much juvenile hormone analog from the treated male that in subsequent mating they infected untreated males with juvenile hormone analogs so that these males now became carriers of the juvenile hormone analog and could sterilize other females. This is not quite the infectious sterilizing agent that Edward Knipling has dreamed of, but it appears promising.

Resistance to Hormone Analogs

IT has been suggested that hormonal insecticides

would have the supreme advantage that it would not be possible for pests to develop resistance to them. However, strong arguments can be advanced that resistance is likely to develop. Consider, for example, juvenile hormone analogs. At certain stages in their development, insects normally inactivate or excrete juvenile hormone and some juvenile hormone analogs (*41, 45*). Thus, nature has endowed insects with a built-in mechanism to resist the artificial application of juvenile hormone and some of its analogs at specific stages. To be sure, the mechanisms which inactivate juvenile hormone and juvenile hormone analogs normally function only at specific times during development. However, the existence of such a mechanism ensures that natural selection could produce populations of insects resistant to exogenous juvenile hormone and its analogs. Similar arguments may hold for the ecdysones, since inactivating mechanisms for these hormones are known.

Now, it may be that this resistance will develop only slowly—perhaps at the negligible rate that broad-leaved plants are developing resistance to 2,4–D. Indeed, the occurrence in plants of phytoecdysones and juvenile hormone analogs suggests that these substances may have served plants as built-in insecticides for millenia. However, insects have a long history of evolutionary success. It appears likely that they will use their variability and reproductive potential to continue to counteract the ingenuity of man and the development of resistance ought not to be ruled out *a priori*, although it may be slow in developing. Any insecticide, hormonally based or not, is bound to act "as a powerful sieve for concentrating resistant mutants that were present in low frequencies in the original population" (*6*). Perhaps attention should be paid to those few insects which appear resistant to some phytoecdysones such as certain sawflies which feed on ferns.

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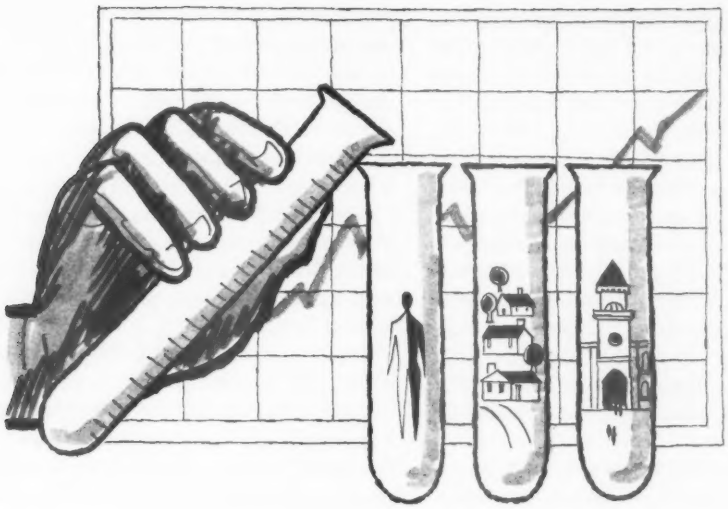
THE AUTHORS (*Continued on inside back cover*)



RESEARCH:
THE THIRD DIMENSION

D. WYNNE THORNE

KNOWLEDGE is the commodity of education. Up to the college level the primary purpose of education is the transmission of knowledge to the student. This knowledge is drawn from a vast and increasing storehouse, and the emphasis is on having students learn established principles. But as his educational experience continues, the student advances to the periphery of knowledge in his area of specialization, and the teacher-student relationship gradually changes. Large libraries become necessary to supplement the teacher and textbook. But this reinforcement often proves inadequate as student and teacher frequently find that recently advanced principles do not satisfactorily account for all the facts.

This can be a critical stage. The educational process shifts from dispensing and receiving knowledge to involving the teacher and student in the evaluation of present knowledge and the discovery of new knowledge. Passage through the transition zone is precarious, because both student and teacher are trading certainty for uncertainty. The student shifts from learning "facts" to mastering the art of discovery; the teacher abandons the authority image and assumes that of a partner in discovery.

At the university level the dimensions of storing and transmitting knowledge remain, but a third dimension is added: that of actively involving students in challenging established ideas and discovering new knowledge through research.

Campus Unrest

SUBSTITUTING uncertainty for certainty is difficult for both teacher and student, and inadequacies in the mechanisms of transition contribute substantially to the prevailing unrest on our campuses. As a result, these are days of soul searching in all institutions of higher education. About 10 percent of

This article was adapted from the author's original paper which was published in the Utah State University Bulletin, Vol. 69, No. 12, Dec. 1969.

such institutions in the United States have had serious student disturbances, and the remainder are asking how they can be avoided or controlled. Many observers doubt that there is general agreement in viewpoint among students. Undoubtedly some demonstrators are alienated and seek to destroy rather than to solve problems. The most universal student grievances, however, are against poor teaching, required courses that seem irrelevant to the student's future, and faculty disinterest in students. The faculty, on the other hand, are unhappy with the average quality and performance of students, and with students who want to monopolize their time with insignificant chatter; and are dismayed by the many demands on their time that limit them in attaining professional goals.

Those seeking reasons for disturbed situations on campuses frequently accuse research of luring teachers away from the undergraduate students by furnishing greater rewards than are attainable from teaching. Articles with such disturbing titles as "Is There a Teacher on the Faculty?", "Publish or Perish," and "While You are Up, Get Me a Grant," have stimulated opposition to research and have led some critics to conclude that if research were banned from the campuses, colleges and universities could return to a cloistered-hall atmosphere.

Relevance and Creativity

BUT the future of universities cannot be built around dreams of the past. The university has become too much a part of the modern world to ever again be isolated from it. Indeed, the complaints of students and faculty are most often against those aspects of the university that have clung too closely to the past. The slogan of today is "relevance." Protests are against the smorgasbord of courses and activities that seem unrelated to the problems of today and the needs of tomorrow, against the shallowness of so much that happens on campus in relation to the depth of human aspirations.

Students and faculty are demanding the right to be creative and to have their creative attempts taken seriously. Adventurous human spirits have been making such demands for as long as history has been recorded. Today's voices differ primarily in that their demands are more general in occurrence, more insistent, and more militant. To be effective, university responses to such demands must promote the truism that creativity and innovation are not accurately measured in terms of merely having an idea. They exist usefully only when coupled with a willingness to work hard enough to test the idea and bring it to fruition.

Around a campus, ideas are a dime a dozen. Really good, mobilized ideas command a much higher price. The need on a campus, as in the world, is for people who can put innovative ideas into action. Valid creativity is a combination of talent, persistence, and energy, perhaps being not so much aptitude as struggle.

Public demonstrations in which students tear up ADP cards symbolize the increasingly widespread demand for recognition of their individuality. This is a worthy demand, essential to the values of a democratic society. But we must use care in interpreting any symbolic actions. Surely no rational person would protest against the machines that free men from drudgery. The protest can only be that while machines have freed men's time so there could be more humanity in man-to-man relations, most of us have not used the time toward that end. In all phases of life men are still too easily indifferent to each other. But this does not justify indifference in a university teaching situation. Any university that acknowledges the significance of the individual is faced with the dilemma that departments do not teach, colleges do not do research, and the student body does not learn. Only the individual does these things. The conditions conducive to learning, teaching, or doing research creatively must be generated within oneself. A university can do many things to promote such internal climates, but it can neither create nor control them.

Student Involvement

ONE way in which a university can encourage personal commitments to constructive, creative efforts is by involving students and faculty in a mutual development and testing of possible solutions to significant problems of today. If the university can help its faculty train and stimulate students so they can and will pursue unfamiliar patterns of thought, the university in effect helps unleash their creative powers. And only by exercising these powers can any individual satisfy the hunger of the human spirit to express itself, the hunger not to be nothing, which is as real and important as the hunger for food. The

teacher-student partnership in creativity, in discovery, in testing ideas, is the secret of effective learning; it is the third dimension of a university. And this third dimension, which occurs most commonly in conjunction with research, is crucial to an individual's learning to be flexible enough to survive in today's turbulent world.

The partnership is learning and the resultant unleashing of creative powers seems to occur most effectively when students and faculty are mutually engaged in discovering and testing possible solutions to important problems. A partnership in discovery makes education a pioneering adventure. The stultifying atmosphere of pointless memorization and busy-work, which too often engulfs the classroom, disappears and excitement takes over. The educational process is no longer a flat surface. The stimulation for learning that grows from involvement in research indicates that instead of too much, there is far too little research on campus. In proper context, research unites rather than divides faculty and student. Research deserves criticism when it becomes isolated from the mainstream of college life. But when properly used to support the educational purposes and to enrich educational opportunities, it is indispensable.

Involvement of university professors and students in research has many ramifications. One is the almost personal tutorial training given large numbers of the best students through participation in research. Another is financial help.

Among the greatest benefits to both students and society from university research are the motivation and training that students receive in the art of discovery. The subject matter of science can be and is taught all over the world in a great variety of institutions and circumstances. The art of discovering new truth, however, cannot be adequately taught in lectures. Mastery of the rules for research rarely suffices to bring the desired results. The art of discovery is most successfully imparted through a master-apprentice association. This is the atmosphere established in the research team consisting of an experienced faculty member and a small group of students varying in training, skills, and research experience.

Impact of Research

THE total impact of research on the quality of a faculty and its effects in improving the environment for learning are difficult to measure. The pattern of research and education is intricate, involving the lives and complex natures of human beings. A university teacher must achieve an extensive and comprehensive understanding of his area of specialization; he must also be able to personally use and effectively present this information to his students and others. Such a task requires the teacher to be thoroughly involved in his specialty and convinced of its importance. The effective university teacher thus must be afflicted with a thirst for knowledge that continually drives him to probe beyond present frontiers.

This craving for the excitement and satisfaction of discovering new knowledge is one reason that few intellectually alive men with the necessary graduate training will consider a university position unless they are assured of an opportunity for involvement in research. For these men the prestige and monetary advantages that may accrue to research have little influence relative to the addictive potency of its intellectual satisfactions. The probing in depth beyond the classroom subject matter enriches the teacher's life and allows him to bring his students in contact with the boundaries of knowledge.

Another seldom publicized aspect of research is its contributions to any university's scope of competence. When teaching and research are directed toward the same goals, they mutually sustain each other in a given institution, and foster the production of graduates equipped to lead happy, useful lives. As the quantity of knowledge increases, and the technological and social complexities of our lives grow, the progress of our free democratic society requires citizens broadly informed on significant issues of the day. If we want a tomorrow, we can no longer be indifferent to pollution, exploitation, and degradation of natural resources; poverty; minority groups; and inhumanity toward the underprivileged. University-trained individuals will have to lead the way.

Man and the Environment

LAND-grant universities have a typical emphasis on man and the environment, which places them in the center of some of the most pressing problems of modern times. Environmental pollution threatens not only the quality and aesthetic aspects of our lives, it endangers the continued existence of man

on earth. This country's growing population and increasing proportion of leisure time enjoyed by the working classes are placing untenable demands and stresses on the environment. The problem is, to a major extent, associated with making wise and optimum use of land and water resources, of climate's effects, and of the associated plant and animal species. A comprehensive understanding of the intricate relationships within natural and man-made environments is the main goal of our extensive program in ecology, and a sub-goal of many of our other programs.

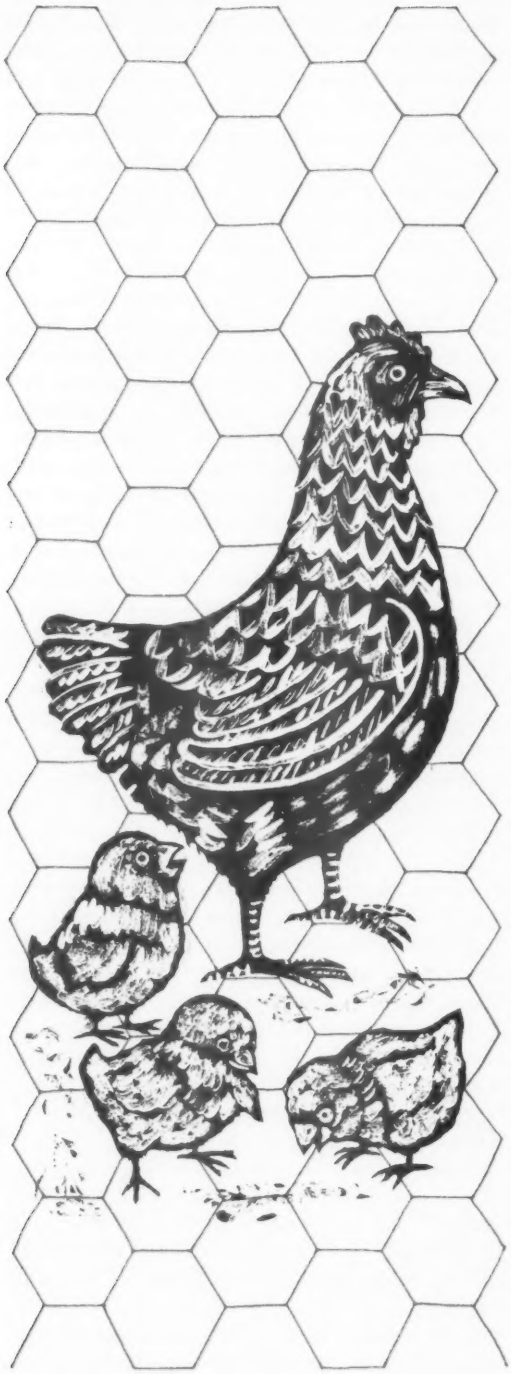
Man's behavior is conditioned by weather, by crowding, by home surroundings, and by clothing—all parts of his personal environment. Thus, not only biological and physical sciences, but art, literature, philosophy, sociology, political science and history contribute to man's understanding of his relationship to his environment. Great writers have portrayed man's dependence on nature. Our violations of the laws of nature, which have endangered the quality of our lives, are rooted in history and are conditioned by our social nature and political structures.

Man's responsibilities toward his environment need careful analysis, definition, and translation into public policy and action programs. The increasing concentrations of people in urban centers, many of whom are mentally and physically deteriorating, constitute the greatest challenge facing this nation today. Restrictions on opportunities to directly experience man's place "within" nature are contributing to a shrinking and distortion of man's concept of himself.

New Approach to Problems

MOST of today's urgent problems do not match the traditional departmental structure of a university. Each problem usually requires the expertise of a wide range of specialists. In order to effectively focus the resources of a university on such problems, some institutions—including Utah State University—have organized task-force groups and centers to coordinate research and training activities. Thus, scientists from a wide spectrum of disciplines—such as chemists, ecologists, geologists, psychologists, and sociologists—may then more easily contribute their diverse talents to specific cooperative ventures. The centers stimulate attention to needed innovations in sequences of courses, and they help bring faculty and students together to discuss, plan and complete research and related programs. The actual teaching of courses and carrying out of research and student training is administered through the departments. This flexible organizational arrangement is proving successful in dealing with many of the significant problems of today.

Without research there would be no university. Without quality research, knowledge would remain static. Without opportunity for creative participation in the discovery of new solutions to significant problems, a university offers little to serious students. But wherever opportunities are deliberately opened up to allow research to reach its full and deserved dimensions, then the university brings an enrichment in depth to the people and the communities it serves.



CURRENT STATUS of the AVIAN LEUKOSIS COMPLEX

MARTIN SEVOIAN

THE uncontrolled proliferation of the precursors of blood cells—commonly termed leukosis—comprises almost all of the neoplastic diseases of chickens. Because these diseases are the greatest cause of mortality and condemnations in chickens in the United States, they have been the subject of much intensive research since the turn of the century. Progress has been slow but encouraging; several new developments show some promise.

Fowl leukosis was the earliest example of a causative virus being involved in neoplasia. Although Caprini (*10*)[1] and Marek (*30*) were among the first to record cases of avian leukosis, the first successful transmission of erythroleukosis with a filterable agent was not reported until 1908 (*22*), thus establishing its cause to be a virus or viruses. Later, Ellerman (*21*) described erythroleukosis and myeloid and lymphoid leukosis in detail. He was able to transmit all three conditions with similar material, which led him to believe that all three conditions were due to a common etiologic agent. Schmeisser (*40*), Stubbs and Furth (*59*) and Olson (*31*) also concluded that erythroleukosis and myeloid leukosis were caused by the same agent and that they may occur simultaneously in a given bird and that either form may change into the other during the course of the disease. Furth (*23*) also reported on a strain of filterable agent which caused lymphomatosis, myelomatosis, and endothelioma in chickens.

[1] Italic numbers in parentheses refer to Literature Cited p. 38.

This paper is a contribution of the Massachusetts Agricultural Experiment Station.

Pappenheimer and his co-workers (*32*) were among the first to give a description of lesions causing paralysis of the legs and wings and to suggest the name of neurolymphomatosis gallinarum. These workers found the predominating lesions to be in the form of infiltrations of lymphoid cells and mononuclear cells, especially in the central nervous system and peripheral nerves. They also noticed similar extensive infiltrations in certain viscera, especially the liver, kidneys, lungs, and the iris of the eye. Tumor formations consisting chiefly of similar cells were also encountered in the ovary and frequently in other organs. In their work, they did not mention the bone marrow, nor did their protocols indicate that they, in any case, made a study of this important tissue. They stated that from their examination of blood smears and from the absence of enlargement of the spleen and liver, the disease they were dealing with was not a form of leukemia. They concluded, however, as the name they suggested for the disease would indicate, that the disease was primarily one of the nervous system.

Burmester and Gentry (*6*) observed that RPL–12 filtrates, originally obtained from the Olson tumor from Massachusetts, caused intravascular lymphomatosis (morphologically indistinguishable from erythoblastosis) with an incubation period of up to 4 months if given in high doses and extravascular lymphomatosis with incubation period up to 9 months if given in low doses. Both pathologic conditions were accompanied by the occasional occurrence of osteopetrosis and rarely fibrosarcomas and endotheliomas.

Cole (*15*) reported a 15-year mortality from leukosis in 2 lines of chickens (K and C lines) selected genetically to be relatively resistant to the disease (2.9 and 3.5 percent respectively). There were nearly 15,000 pullets in each strain. In both lines, the losses from the visceral form were somewhat greater than those from neurolymphomatosis, while losses from the ocular form were relatively few. In contrast to the low mortality of K and C lines, the level of mortality from leukosis in more than 9,000 S-strain chickens, genetically selected for susceptibility to the disease, was 39.5 percent where the neural was several times as frequent as visceral lymphomatosis. The level of the visceral form, 5.7 percent, was about twice the total leukosis loss in the resistant (K and C) strains and was, no doubt, a minimum because much of the neurolymphomatosis mortality came at an age prior to that at which the visceral form is more apt to express itself. A few cases of erythroleukosis and myeloid leukosis were also found. During the same period other forms of neoplasms such as fibromas, myxomas, fibrosarcomas, gliomas, adenocarcinomas were observed which were found to be highest in incidence in the S-line and lowest in K-line.

Etiology

SOME early investigators of avian leukosis tended to believe that each transmissible strain of virus was an etiologic unit, whereas others assumed that a single agent was responsible for all the pathological signs of the avian leukosis complex. Recent developments have begun to relate and delineate various parts of the avian leukosis complex and have indicated that both groups of investigators were partially correct. Three different types of viruses have been investigated and their relationship to the complex is herewith presented.

Type I Viruses (lymphoid leukosis viruses)—Rubin (*35*) discovered in chicken embryo fibroblasts a noncytopathogenic virus that he called RIF, an acronym for resistant-inducing-factor. Cells in tissue cultures infected with RIF virus were found partially or completely resistant to challenge with Rous sarcoma virus (RSV) which normally induces changes in cell morphology leading to a piling up of altered cells to form foci. This interference phenomenon allows *in vitro* assays. Additional research suggested that RIF virus is a naturally occurring leukosis virus which is indistinguishable from the virus of visceral lymphomatosis (RPL–12) in its physicochemical, immunological and biological characteristics. Furthermore, it has been shown that four established strains of visceral lymphomatosis and an avian myeloblastosis virus can be detected in tissue culture by interference with RSV (*37*). Thus, RIF activity or the lack of it denotes a differential characteristic among viruses but it does not necessarily imply antigenic relationships. Data indicate that horizontal transmission of RIF virus is slow. The viability is not affected by freezing, and the virus appears to be moderately antigenic (*36, 38*).

Little has been reported on the pathological response of the chicken to RIF virus. However, birds

from which RIF virus was isolated commonly had a lymphomatous involvement of the liver and spleen and occasionally the ovaries, kidneys, lungs, and intestinal organs. Also, neurolymphomatosis and osteopetrosis were found on occasion in birds with RIF virus or possessing virus with RIF activity (*38*). Other leukosis viruses possessing RIF activity such as RPL–12 have been reported to have a wider oncogenic spectrum causing many different types of tumors, whereas the myeloblastosis virus has not been as variable in its cellular response (*24*). Although the sensitivity of RIF virus to the genetic constitution of the chickens has not been reported, its indistinguishable biologic characteristics from RPL–12 would indicate that it is sensitive to various genetic lines of chickens (*5*).

Susceptibility or resistance to infection by viruses of the A and B sub-groups is controlled in each case at a single genetic locus; the gene for susceptibility is dominant over the gene for resistance (*18, 33*). By testing cells, embryos, or chicks for response to A or B sub-group RSV, it is possible to forecast their response to Type I leukosis virus. Strains of RSV are now being used in this way by commercial poultry breeders in progeny tests for selecting chickens genetically resistant to leukosis infection.

Type I lymphoid leukosis virus is widespread in poultry populations and is excreted in the droppings and saliva of diseased birds and normal-appearing carrier birds. This virus is fragile and it is doubtful whether it normally survives outside the chicken for more than a few hours. Both A and B sub-groups of Type I leukosis occur in the field, although most published work relates to the A sub-group. When susceptible chickens are exposed to Type I viruses, they develop specific virus-neutralizing antibodies which may be detected by their ability to neutralize RSV of the same sub-group as the infecting Type I virus.

Chickens are most susceptible to contact infection during the hatching and brooding period. Those from immune birds carry maternal antibody for the first few weeks of life which provides them with partial immunity to contact infection. Some immune birds also carry the virus and pass it to a proportion of their offspring through the egg, and these infected embryos become immunologically tolerant. When these embryos hatch they are viraemic, and are unable to develop an active immunity, but show no signs of their infection. At maturity, female viraemic chickens transmit the infection to all their offspring, which in turn become viraemic and tolerant. There is no evidence for congenital transmission of infection by the male.

An infected flock thus consists of non-infected immune birds, infected immune birds, and a small proportion of tolerant viraemic birds; in addition it appears that non-infected, non-immune birds may also be present. Only some of the two infected classes develop leukosis and the remainder act as virus carriers. Genetic factors are believed to influence whether or not an infected bird develops leukosis. Losses due to Type I infections are estimated at 20 percent of all losses attributed to the leukosis complex.

No methods of treatment or vaccination against Type I lymphoid leukosis are available. The disease must be controlled by restricting spread of infection by suitable flock management and by the use of genetically resistant stock. Young stock should be reared in isolation from adult stock and other sources of infection. To minimize losses following egg transmission of the virus, stock should be obtained from a flock with an absence or low incidence of Type I viruses. If possible, chicks from several parent flocks should not be mixed because of the possibility of introducing strains of Type I infection against which a proportion of the mixed chicks may have no immunity.

Type II Viruses (Marek's)—In 1962 Sevoian *et al.* (*51*) reported the isolation of a field virus (JM) which was filterable through a 0.3 micron filter. This virus produced visceral, neural, and ocular lymphomatosis with cellular and cell-free inocula in a susceptible (S) line of chickens within a 21-day incubation period. Spiewak (*58*) produced similar lesions in S-line chicks with plasma centrifuged at 71,000 xg from JM-infected chickens. This virus has been grown in a number of different cells in culture (*14, 56*) and has been shown to produce cytopathic changes in chick kidney cells and duck embryo cells. Using whole cells collected from infected chickens to inoculate cultured cells, these workers have demonstrated a herpes type virus with electron microscopy. More recently, Calnek and Hitchner (*8*) demonstrated similar viral particles with cell-free material taken from skin of JM-infected chickens.

The primary isolation of Type II viruses can also be done in chicken embryos. Sevoian (*47*) reported a primitive mesenchymal cell response particularly in the liver, spleen, chorioallantoic membrane (CAM) and yolk sac of embryos, inoculated via the yolk sac at 4 days of incubation with JM-infected blood cells from chickens and JM-infected duck embryo fibroblast cultures (25th passage) whereas embryos similarly inoculated with non-infected control cells remained normal. In similar trials, von Bulow (*61*) reported the formation of pocks on the CAM.

Infection levels with Type II viruses in commercial flocks often approach 100 percent (*46*). It is estimated that Type II infections are responsible for approximately 75 percent or more of the losses attributed to the leukosis complex. The levels of viremia and antibodies appear to be higher in susceptible lines of chickens than in resistant lines (*48*). Also, levels of viremia fluctuate considerably within a given bird. Infection of chickens with Type II viruses induces chronic viremias as well as the production of various types of antibodies which can be detected by the agar gel double diffusion technique (*11*), the florescent antibody technique (*34, 57*), and the hemagglutination (HA) technique (*20, 27, 65*).

The usefulness of the HA test for quick and low-cost screening of a large number of samples is indicated, although the indirect HA test using sensitized cells with antigen or antibodies is much more specific. Chubb and Churchill (*12*) have reported that the presence of precipitin antibodies gave a significant protective effect against morbidity and mortality to injections at one day of age to Type II (Marek's) viruses, but the protective effect was not significant during natural exposure after antibodies waned presumably at 1 to 3 weeks of age. On the other hand, Sevoian (*48*) has found genetic resistance to be a more dominant and lasting factor when groups of susceptible (S) and resistant (K) lines of day-old chicks, with comparable levels of antibodies, were naturally or experimentally exposed to JM virus and where S-lines succumbed and K-lines survived 100 percent or nearly so. Using a non-contagious and presumably non-infectious JM virus (JM-V), Sevoian (*45*) challenged day-old off-spring with and without parental antibodies to demonstrate neutralizations (protection) up to 1,000 times as compared with non-immunized controls. Similarly, constant and heavy natural exposure to JM virus of chicks with antibodies resulted in 80 percent protection to 8 weeks of age as compared to 50 percent in controls.

The host response to JM virus in cell suspensions administered by the parenteral route to young S-line chickens paralleled the clinical signs and lesions observed in the neural, visceral and ocular forms of leukosis in field infections (*50*). When JM virus was administered to three successive generations of S-line chickens at the same time, the clinical response resulted in a gradient decrease of neural and ocular involvement from a high in young chicks to a low in mature birds which predominantly had the visceral form. This distribution of signs and lesions in the various age groups paralleled that of field infections.

Experimentally, JM virus has also produced visceral, but not neural leukosis in turkey (*42*). Histogenetic studies using cell-free inoculum indicated that the offending lymphoid cells originated from the primitive mesenchymal cells around capillaries and of the tunica adventitia of the arterioles, neurilemmal cells and the lining cells of the hepatic sinusoids in descending order of incidence. JM virus, however, had a predilection for the medullary cells of the bursa of Fabricius and also for a variety of epithelial cells, especially in the kidney tubules and the follicular epithelium of the feather follicles (*8*). Dander shed by the skin was infectious (*2*).

Studies on epizootiology indicate that the airborne route of infection (*52*) and direct contact (*4, 63*) are the principal means of spread. Insect vectors have been incriminated but probably are not a significant means of spread. Evidence suggests that the virus is transmitted via the embryo (*46*). However, subsequent studies using the agar gel precipitin test (*64*) and the indirect hemagglutination test (*65*) have not demonstrated virus transmission via the embryo. Similarly, congenital transmission of antibodies has been reported (*62*) whereas significant levels of antibodies in the yolk sac or blood of day-old chicks from exposed dams have not been found (*55*).

Virulent (JMV) and non-virulent (HPR-14, JM) strains of Type II viruses have been used for vaccine studies. JM–V strain was demonstrated to be antigenically related to JM strain (*27*) and stim-

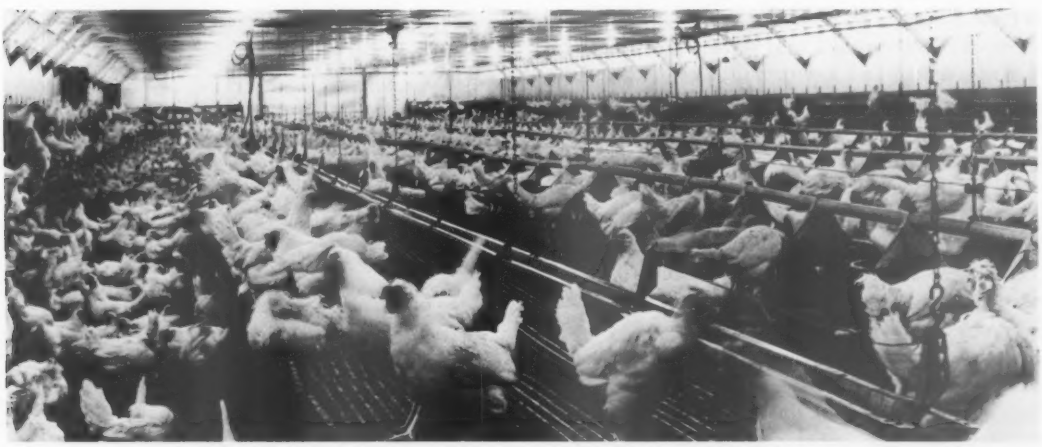
ulated neutralizing antibodies in dams which was readily transmitted via embryo to offspring affording them some protection during early life against natural exposure to JM strain (*46*). When tissue-culture propagated strains of Type II viruses were injected into day-old chicks, they afforded good but incomplete protection against the development of tumors, but not against the infection (*14, 28*). Similar results have been reported with a herpes virus isolated from turkeys (*64*).

Genetic selection for resistance to Type II leukosis has been demonstrated (*16*). In only two generations of selection of sire families, the response of progeny to inoculation with JM strain of a selected resistant strain was 13 percent whereas the susceptible line was 91 percent within an 8-week period.

Type III Viruses—In 1964, Sevoian, Larose and Chamberlain (*53*) reported the highly lethal characteristics of T virus originally isolated by Twiehaus of Manhattan, Kansas. This virus was capable of killing within 2 weeks 100 percent of birds from various genetic lines. Clinical nervous or ocular signs were not observed. Affected chicks manifested greatly enlarged lymphomatous livers and spleens with sub-scapsular grayish-white focal nodules irregular in shape and ranging up to 1 cm. Extensive tumor nodules were also seen in the gonads, heart, kidney, and other visceral organs. The virus elicited abundant proliferation of lymphoid cells within the tunica adventitia of the small arterioles resulting in multiple lymphomas throughout the tissues. In addition, "hyperplasia" of the cells lining the hepatic sinusoids was extensive and widespread, generally involving the entire lobe. Turkeys and Japanese quail were affected similarly with T virus (*60*). Recently, Levine has demonstrated tumors in infected hamsters. The viability of the virus was adversely affected by freezing (*44*).

A preliminary trial indicated that the virus possessed no RIF activity (*43*) but additional trials are underway to determine this characteristic. T virus was highly antigenic in chickens (*29*). T antiserum did not neutralize JM virus using S-line chicks as the indicator host (*43*). Horizontal spread of T virus among contact birds was slow (*29*). Growth of the virus in various genetic lines of chickens was approximately the same (*53*). Ziegel *et al.* (*66*) reported that T strain was an RNA virus.

The pathological response of four genetic lines of embryos to T virus centered on its ability to stimulate the primitive mesenchymal cells in the hematopoietic tissues found in or around the walls of the capillaries, arterioles, and sinusoids of the liver and bone marrow. The process was an extravascular lymphopoiesis and myelopoiesia. A pronounced pathological response could be detected by gross examination of the embryos as a result of inoculation of cell-free virus preparations into the yolk sac. When serial dilutions of virus were inoculated, a graded pathological response was evident, consist-



Neoplastic diseases are the greatest cause of mortality and condemnation in chickens in the United States. Annual losses are estimated at $200 million.

ing of transcoloration and enlargement of the livers and spleens. This effect was neutralized by serum and egg yolk from experimentally immunized chickens with T virus (*29*). Although the economic significance of T virus is largely unknown, preliminary surveys (*41*) of chicken flocks indicate a low incidence.

To determine whether T virus could grow in embroyonic tissues *in vitro,* serial passages in two trials were conducted by inoculating 24-hour primary fibroblast cultures from embryos of a hen previously tested and repeatedly found free from RIF virus (*29*). The assay for RIF virus was performed according to the method described by Rubin (*35*). At the end of 4 serial passages, three 1-day-old S-line chicks were inoculated intraperitoneally with 5 x 10^5 cells of the inoculated line and another 3 S-line chicks were inoculated with 5 x 10^5 cells of the control line cells. Both tissue culture trials revealed that the T virus can grow in serial passage of chick embryo fibroblasts and still retain its pathogenicity in chicks after 4 passages. Although the virus demonstrates a capability for growth in chick embryo fibroblasts, no evidence of cytopathic alteration was observed in unstained cell cultures.

Terminology and Classification

RECENT advances in studies of the causes and pathogenesis of a group of neoplasms heretofore known as the avian leukosis complex have provoked a reconsideration of established terms and classifications. Several classifications (*1, 3, 9, 13, 17, 25*), each a product and a contribution of its time, have been published in the literature. Some have implied an etiological separation, whereas others have been a clinical and pathological classification without etiological inference.

As more is learned about the cause of tumors, it would seem desirable on first consideration to incorporate within neoplastic disease classifications the distinctive etiological separation of tumors. In workable considerations, however, a disease classification would be best assimilated and integrated if it were first pathologically oriented and then etiologically tempered. If a classification were etiologically oriented, like tumors would be placed in different groups and unlike tumors would be grouped together. The reason is that different etiological agents can cause the same kind of tumor, as with RIF, JM and T viruses (*36, 51, 53*), and a single agent such as RPL–12 (*7*) can cause many pathologically different kinds of tumors of various cellular compositions. The grouping of unlike tumors caused by single etiologic agents would make difficult if not impossible their assimilation into existing tumor classification. Furthermore, it is not currently known whether other etiological factors such as radiation or carcinogens can cause these neoplasias, so that a given kind of tumor might be multi-etiologically oriented, which again would confound such a classification. The most appropriate approach at this time, therefore, is to integrate etiological considerations into a pathologically-oriented classification, with incorporations of universally understood terms.

In the light of these considerations, the following open-ended flexible classification is proposed, wherein types and subtypes of causal agents may be added as needed.

Avian Leukosis Complex

By: Sevoian (*45*)	By: Biggs (*3*)
LYMPHOID	
Type I (Myxovirus)	Lymphoid Leukosis
Subtype A	
B	
C	
Type II (Herpesvirus)	Marek's disease
Type III (Myxovirus)	?
MYELOID	
Type I	Myeloid
ERYTHROID	
Type I	Erythro
NON-LEUKOSIS TUMORS	LEUKOSIS TUMORS
Type I	Osteopetrosis
Osteopetrosis	
Fibrosarcomas	
Endotheliomas	
Nephroblastomas	
Etc.	

It is considered that the framework of this hemopoietic classification is pathologically inclusive and that it is etiologically open ended. This classification

is purposely open ended to accommodate changes or additions of an infinite number of types and subtypes of agents. A virus classification, which is distinct from the proposed disease classification, will follow when more is known about the causal agents. Arbitrarily, the RIF-type avian tumor viruses, having an inhibitory effect upon the growth of Rous virus *in vitro* fibroblasts and having a common antigen as determined by the COFAL test (*39*), has been designated as Type I, with respective subtypes, and placed in appropriate cellular categories. This group of viruses can stimulate the lymphoid, myeloid, and erythroid blood lines. In addition, they may cause fibrosarcomas, endotheliomas, osteopetrosis, and nephroblastomas. Therefore, a separate category of non-leukotic tumors considered pathologically peripheral to the confines of the leukosis classification was made to designate types of viruses demonstrated to have such multipotent oncogenic spectra.

A second major category of agents causing lymphoid tumors (so-called Marek's disease) is that group (Type II) which is represented by JM (*51*) and B–14 (*4*) types; these strains do not have RIF activity (*43*). Although the PMC are predominantly proliferated by this group of agents, Schwann cells are also stimulated. Antigenically this type of virus (JM) is different from those of the RIF-type (*44*). A third types of virus causing leukosis in poultry is represented by T virus (*53*), which has been propagated in tissue culture and has not been shown to possess RIF activity (*53*). T virus has been shown to be antigenically different from JM-type and Rous viruses and morphologically different from the RIF-type viruses.

It is generally agreed that the cell types of lymphoid tumors (arising primarily from the primitive mesenchymal cells or stem cells) stimulated by each of these agents are indistinguishable. By pathological description and definition the neoplasm caused by each of these agents is a lymphoid leukosis. In addition, the RIF-type viruses cause myeloid and erythroleukosis as well as several non-leukotic neoplasias. The term lymphoid leukosis, therefore, is especially applicable to the more consistent lymphoid response of the JM-type and T-type viruses, but also applicable to the less consistent lymphoid of the RIF-type viruses—not because these responses cannot be histologically differentiated but because they do, in fact, each elicit a lymphoid leukosis. Hence, a chicken having neoplasia heretofore known as neural, visceral, or ocular lymphomatosis (*1*) or Marek's disease (*4*) caused by the JM-type agent would, under this classification, be termed Type II leukosis or, more specifically, Type II lymphoid leukosis. Or if a bird had visceral lymphoid leukosis without the presence of Type II virus (JM-type), it would be termed Type I or III lymphoid leukosis, depending upon the presence or absence of one of these types.

Critical Appraisal and Outlook

TO date, the greatest strides experimentally for control of Type II leukosis have been made in the genetic selection of resistant stock. For example, it is known that resistant (K) lines have lower virus titers than susceptible (S) lines but not all infected chickens develop the disease. Therefore, low or nonviremic exposed stock indicates its ability to resist or cope with the infection and should be so selected with the aid of a method indicating viral titers and possibly antibodies. The criterion of the presence or absence of signs or tumors of leukosis at 8 weeks of age is relatively crude and expensive, and more sensitive economical laboratory techniques should be sought.

Chickens reared in isolation or housed with filtered air generally receive a minimum of exposure or infection but, depending upon genetic susceptibility and sometimes physical environmental accidents, the method is undependable and by itself is one of less promising alternatives. When coupled with a vaccination or genetic selection programs, however, this environmental technique could have more value.

Considerable speculation has been created by recent data on the prospect of a vaccine. The experimental vaccines when given at an early age have had a depressing effect on the development of tumors, at least during the first few weeks of life when chicks have not received early natural field exposure. Field trials reported by Eidson *et al.*, at the 1970 A.V.M.A. meeting in Las Vegas indicate vaccinated chicks received little or no protection to leukosis from the tissue-culture propagated GA strain and the cell-free turkey herpes virus and only moderate protection with the cellular turkey virus. This condition may have been due to early field exposure

after vaccination or possibly improper dosage. It is estimated that the vaccine will cost about 17 cents per bird.

By far the most serious disadvantage thus far reported is that all of these mild or attenuated vaccines stimulate little neutralizing or protective antibodies and usually result in a chronic or persistent viremia due to the vaccine virus; in addition, the vaccine does not prevent Type II or Marek's disease infections from entering the vaccinated chicken. Thus, it is a system which has an economic cosmetic value. A satisfactory live vaccine is one which causes a temporary mild form of the disease, stimulates protective antibodies which then are able to neutralize the infection within the bird, thereby resulting in a negative or clean chicken. Because JM–V strain is highly antigenic in producing neutralizing antibodies and reportedly does not possess a virus to produce a chronic infection, it appears that the use of this strain would be a preferable antigen for vaccination against leukosis.

Some encouraging results (unpublished) have been gained through the use of chemical compounds in inhibiting the growth of tumors in chickens infected with JM virus. If results continue to be sufficiently encouraging, field trials will be conducted.

If further gains are to be expected in lessening the heavy economic losses to the poultry industry, it seems likely that future research on the avian leukosis complex might well concentrate on the following areas of priority:

1. Eradication techniques and programs directed toward the genetic selection of resistant low- or non-viremic stock.
2. Development of a vaccine which produces *immune, non-viremic* chickens.
3. Studies on transmission of Type II infections to animal species other than poultry.
4. Screening of drugs which reduce the incidence of infection or disease.
5. Studies on minimizing environmental horizontal transmission (isolation rearing or air filtration).

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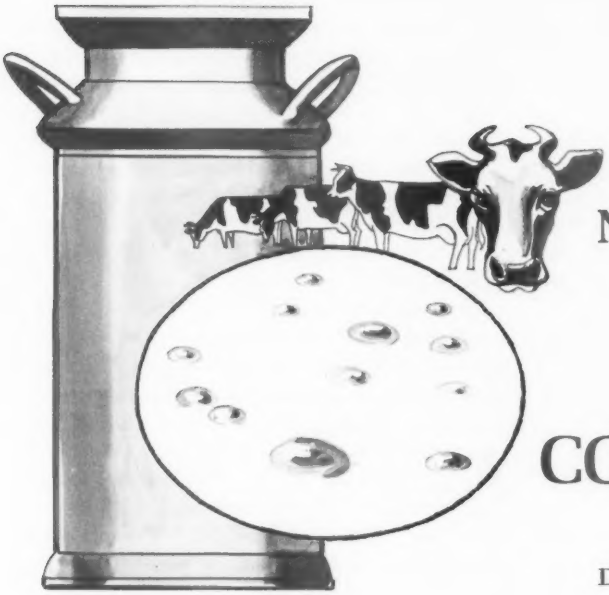

New Micromethods for Isolating and Characterizing LIPID CONSTITUENTS

DANIEL P. SCHWARTZ

RESEARCH in the field of lipid analysis has accelerated at such a rapid pace in the last decade that several new journals devoted exclusively to the subject have been created. The impetus given to the field must be directly attributed to gas-liquid partition chromatography and to thin-layer adsorption chromatography. Although these elegant methods have become almost indispensable to the laboratory, they are essentially micro separation techniques of limited capacity and must usually be supplemented by additional isolation and identification methods, especially when a highly complex material is being analyzed. For about the past 10 years, the Dairy Products Laboratory of the Agricultural Research Service, USDA, has been developing new and improved methods for isolating and characterizing constituents of natural products with emphasis on lipids and especially on milk fat. The methods were developed with speed, convenience and simplicity in mind so that laboratories without elaborate equipment could undertake similar research in this field.

An important feature of virtually all methods discussed in this paper is the utilization of an inert support impregnated with one of the reactants dissolved in an aqueous phase. The other reactant, dissolved in a water-immiscible solvent, is then passed over the column, reaction taking place on the surface of the coated support. These two-phase column reactions have been found to be extremely efficient for reacting micro quantities of specific classes of compounds present in very dilute solution. Besides requiring minimal manipulation, they are usually faster to run, and reactions tend to go to completion because of the high molar ratio of one reactant to the other and the continuous removal of the products from the reaction medium.

Analysis for Carbonyl Constituents

THE program for developing new analytical methods received its initial impetus from a need to isolate and identify off-flavor constituents and their non-volatile precursors in milk and other dairy products. One of the flavors which has been particularly troublesome and causes considerable economic loss is the so-called oxidized flavor. This flavor is the result of oxidation of unsaturated fatty acids by oxygen under metallic catalysis which ultimately results in the appearance of carbonyl-compounds—some of which impart the typical "cardboard" or "tallowy" taste characteristic of oxidized flavor.

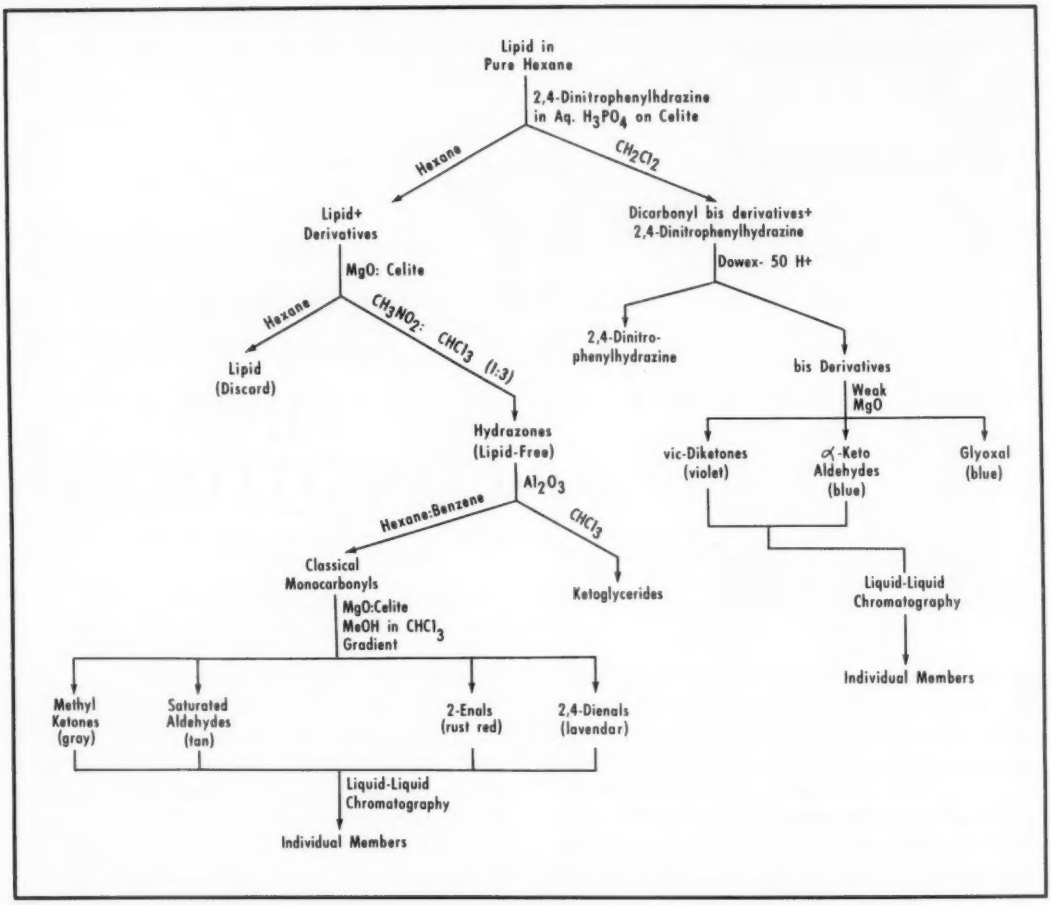

FIGURE 1—Quantitative isolation and characterization of carbonyl compounds from lipids.

The problem of isolating and identifying the carbonyls in oxidized fat, classically approached by steam or vacuum distillation, was instead performed by direct reaction of the carbonyl compounds in the fat with 2,4-dinitrophenylhydrazine mediated by a column of Celite impregnated with an aqueous acidic solution of the reagent (*19*).[1]

The direct isolation approach was thought to have two major advantages over distillation: (1) both volatile and non-volatile carbonyl compounds would be derivatized and eventually isolated, and (2) the method ostensibly would lend itself more suitably to routine analysis. As it turned out, both convictions were upheld, although the former resulted in several unanticipated problems which will be discussed later.

The flow diagram depicted in figure 1 gives the various steps in derivatizing, isolating, fractionating, and identifying the carbonyl compounds in a lipid. An important step in the procedure is the separation of the complex mixture of derivatives into classes, that is, into methyl ketones, saturated aldehydes, 2-enals and 2,4-dienals. Fortuitously, each class displays a characteristic color on the adorbent, thus better enabling the analyst to tentatively classify a band and to "cut" fractions from the column.

[1] Italic numbers in parentheses refer to Literature Cited p. 49.

Although initial interest was focused on the classical monocarbonyl fraction resulting from fat oxidation, a number of observations made in the course of the work eventually led to methods for analyzing dicarbonyl compounds as bis(2,4-dinitrophenylhydrazones) in a similar systematic fashion (*10, 25*). These are included in the schematic of figure 1.

Application of the methods to fresh butteroil uncovered a number of hitherto unrecognized components of this lipid, the main one being the ketoglycerides. These are glycerides containing a keto acid esterified to glycerol along with two fatty acids. They were found to occur in milk fat in a concentration of 5 to 15 μmoles/g. The keto group was distributed in all positions of the chain but mostly in the 9 and 10 positions. Ketopalmitic and ketostearic acids were found to be the predominant keto acids in milk fat (*4*). The presence of this class of carbonyl compound in such relatively high amounts in milk fat was one of the unforseen problems in the isolation of traces of "developed" carbonyls.

Investigation of other lipids revealed that the animal fats (lard, tallow) and vegetable oils (corn, olive, safflower) and nut oils (walnut, peanut) which were examined contain substantial amounts of the ketoglycerides.

The formation and biological significance of ketoglycerides in lipids has not as yet been elucidated, but the method for carbonyl analysis can furnish an accurate estimate of this structure.

One can visualize a possible role of the ketoglycerides in food products, although it is somewhat speculative. The interaction of amino groups of proteins with the carbonyl group of the ketoglycerides should theoretically occur under the proper conditions, possibly resulting in a protein-ketoglyceride complex with peculiar solubility characteristics. Another reaction leading to the possible formation of α-ketoaldehydes in lipids would be the oxidation of unsaturated keto acid-containing glycerides possessing the ene-2-one structure. The ene-2-one linkage undoubtedly exists in lipids and especially in those of plant origin.

The β-ketoglycerides deserve special mention since they are the source of methyl ketones arising in many dairy products. By using the methods described, it was possible to accurately quantitate the amount of β-ketoglyceride in milk fat via the methyl ketones formed when milk fat is heated in the presence of water. The molar ratio of the ketones was also established, as was the effect of temperature (*20*) and water content (*26*) of the fat on ketone formation.

The method also led to the recognition that "bound aldehydes" were present in milkfat. These aldehydes are generated from a vinyl ether-type linkage under acidic conditions. The 2,4-dinitrophenylhydrazine column, being at a pH of approximately 1.5, readily liberated these aldehydes which were quantitated, isolated, and purified as the derivatives. The parent aldehyde was then regenerated from the derivative and separated by gas-liquid chromatography. In this instance, it was again a simple matter to quantitate the aldehydes and thus establish the concentration of the alk-l-enyl ether glycerides that are present in milk fat (*5*).

Another carbonyl component of milk fat which was isolated for the first time in nature was identified as Δ^7cholestene-3-one. It was isolated as its 2, 4-dinitrophenylhydrazone directly from the fat, and, after purification, was regenerated to give the parent compound which was identified by its mass and infrared spectra and melting point (*8*).

Application of the methods to the analysis of carbonyl compounds in some off-flavored dairy products has been made. The sequence of the appearance of carbonyls as milk oxidizes was established quantitatively (*6*). The oxidized flavor was not usually detectable organoleptically until the chemical analysis revealed the presence of alk-2,4-dienals.

A carbonyl compound which contributes significantly to the flavor of stored, dry, whole milk was identified as o-aminoacetophenone. This compound, which has a grape-like odor, can be detected by taste in skim milk at a level of 0.4 part per billion (*7*).

An interesting carbonyl compound that develops in the drying of whole milk in the summertime, but not at other times of the year, was identified as trans-6-nonenal. This compound, which is mainly responsible for the so-called "dryer" flavor of whole milk powder, is an extremely potent flavor compound and can be detected organoleptically at a concentration of 0.07 part per billion (*9*).

Analysis for Hydroxylated Constituents

THE successful application of the carbonyl methods to milk fat and other lipids prompted the de-

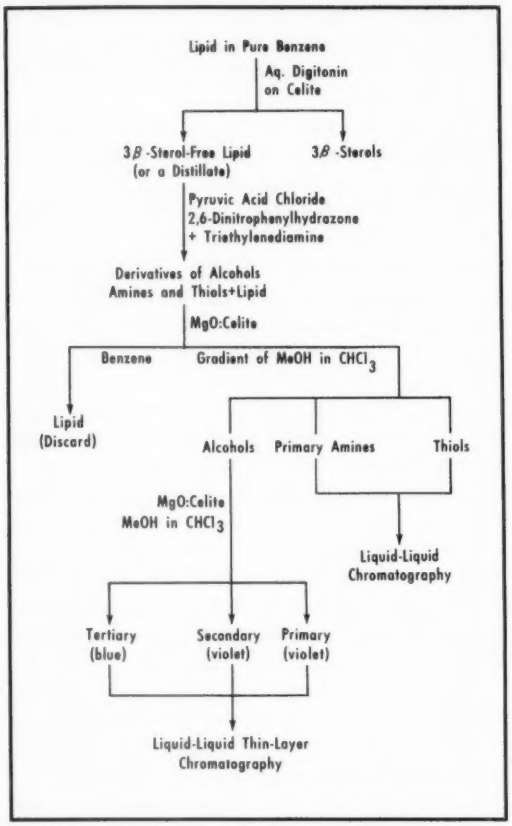

FIGURE 2—Schematic for isolation, fractionation, and identification of alcohols, amines, and thiols from lipids.

velopment of an analogous method which might be used to determine alcohols, amines and mercaptans in lipids. A major flavor defect encountered in stored, dry, whole milk is staling—an off-flavor resulting from a combination of compounds which develop over time. The major contributors to the stale flavor are the Δ- and γ-lactones, which are thought to arise from the 4- and 5-hydroxyacids originally esterified in the glycerides.

A new acid chloride reagent, pyruvic acid chloride 2,6-dinitrophenylhydrazone, was synthesized and found to react instantaneously and quantitatively with a large variety of alcohols and also with primary and secondary amines and mercaptans (*11*).

Fig. 2 is a schematic depicting the various steps in forming, isolating, fractionating, and identifying the various reactive classes either directly from a lipid or from a distillate. Note that in the direct reaction of the acid chloride with the lipid, cholesterol—because of its high concentration in milk fat and some other lipids—is first removed very simply by using a column of Celite impregnated with digitonin. This procedure has been very useful; its applications are discussed later. From this step on, the procedure is very similar to the carbonyl method in figure 1. The alcohol, amine, and thiol derivatives are first separated from each other (*15*). Then the three classes of alcohols (tertiary, secondary, and primary) are subsequently separated from each other and the individual members of each class are resolved by thin-layer partition chromatography (*14, 16*). Primary and secondary amine derivatives are fractionated in a similar manner (*12*). All fractionations are aided by some color differences between classes.

Application of the method to milk fat has furnished an accurate estimate of the major classes of hydroxyl-containing compounds (for example, the hydroxy acid glycerides, diglycerides, cholesterol and some of the minor sterols).

Utilization of pyruvic acid chloride 2,6-dinitrophenylhydrazone for isolating a number of minor sterols from milk fat is depicted schematically in figure 3. In order to facilitate their isolation, especially for quantitative analysis, it was necessary to develop a new technique for saponifying lipids, that is, one which avoided the use of alcoholic solvents for dissolving the alkali and the lipid. This method, like the digitonin column method, has appreciably aided the analysis of lipids and will be elaborated on further in this report.

By using the method outlined in fig. 3, it was possible to isolate, identify, and quantitate lanosterol and dihydrolanosterol (*1*) and also to positively identify β-sitosterol as a normal constituent of milk fat. β-sitosterol was isolated from the digitonin column by regeneration of the 3β-sterol-digitonin complex with dimethyl sulfoxide. This technique also forms the basis for a method of detecting milk fat adulteration by vegetable oils (*3*).

The method for isolating alcohols has revealed the presence of a large number of complex alcohols that are normal constituents of milk fat. No simple alcohols, that is, long chain wax alcohols, have been detected in the unsaponifiable fraction of milk fat.

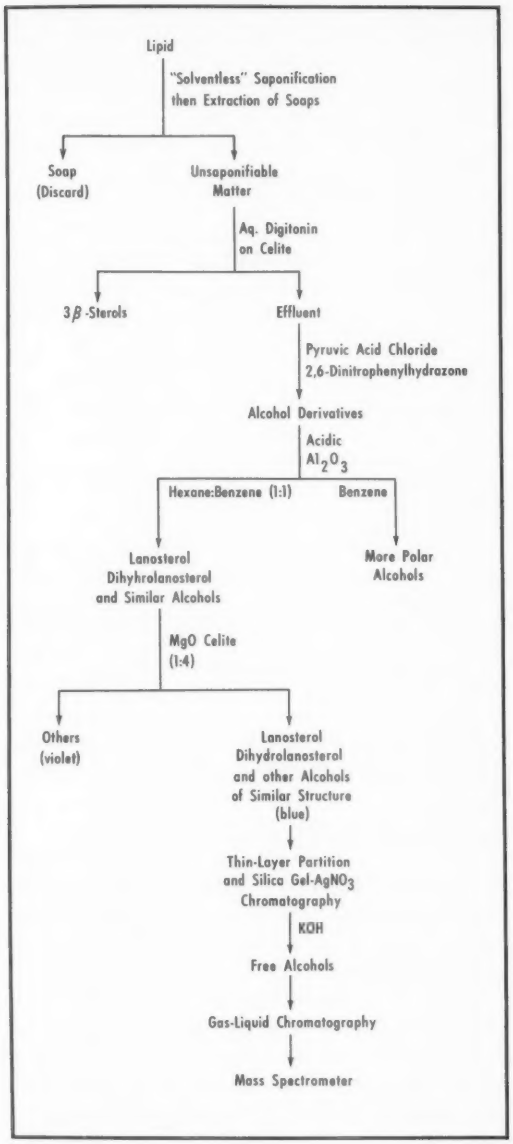

FIGURE 3—Isolation of lanosterol, dihydrolanosterol and related alcohols from lipids.

Periodic Acid Column Oxidation

THE well-known periodic acid reaction for the oxidation of vic-glycols and similar structures is difficult to apply to lipids because of the problem of finding a suitable mutual solvent for the lipid and the oxidant. A convenient procedure was therefore developed which circumvented this situation (*24*) and greatly facilitated a study of some of the structures susceptible to periodic acid oxidation. These include the 1-monoethers of glycerol and the 1-monoglycerides.

A benzene or methylene chloride solution of a susceptible structure is passed over a small column of calcium or magnesium sulfate impregnated with a saturated aqueous solution of periodic acid. The oxidation products, usually aldehydes or acids, emerge at the exit end of the column and can be detected by gas chromatography and supplemental techniques; or the aldehydes can be derivatized and determined qualitatively and quantitatively.

Application of the periodic acid column to the unsaponifiable matter of milk fat has facilitated a thorough quantitative study of the 1-glyceryl ethers which are present in small amounts in milk fat. In another application, the 1-monoglycerides were quantitatively determined. Both procedures are described later.

An interesting facet of the periodic acid reaction was revealed during the development of the method. It was found that no oxidation of susceptible structures took place if the periodic acid solution was impregnated onto Celite or onto fine glass beads, which indicated that silicates might inhibit the reaction. Another interesting observation was that formaldehyde could not be detected in the effluent of the column oxidation of either of the above classes.

Determination of Glyceryl-1-Alkyl Ethers

GLYCERYL-1-alkyl ethers (glyceryl ethers) although known for about 50 years, have only lately been extensively investigated. These recent studies, almost exclusively done by gas-liquid chromatography, have indicated that glyceryl ethers may be expected to be found widely distributed in the tissues of fish and animals.

In order to more thoroughly investigate the glyceryl ethers occurring in milk fat and other lipids, a procedure was developed for the quantitative isolation of the glyceryl ethers and their subsequent fractionation and characterizations (*21*). This procedure, shown schematically in figure 4, utilized no instruments except a spectrophotometer and

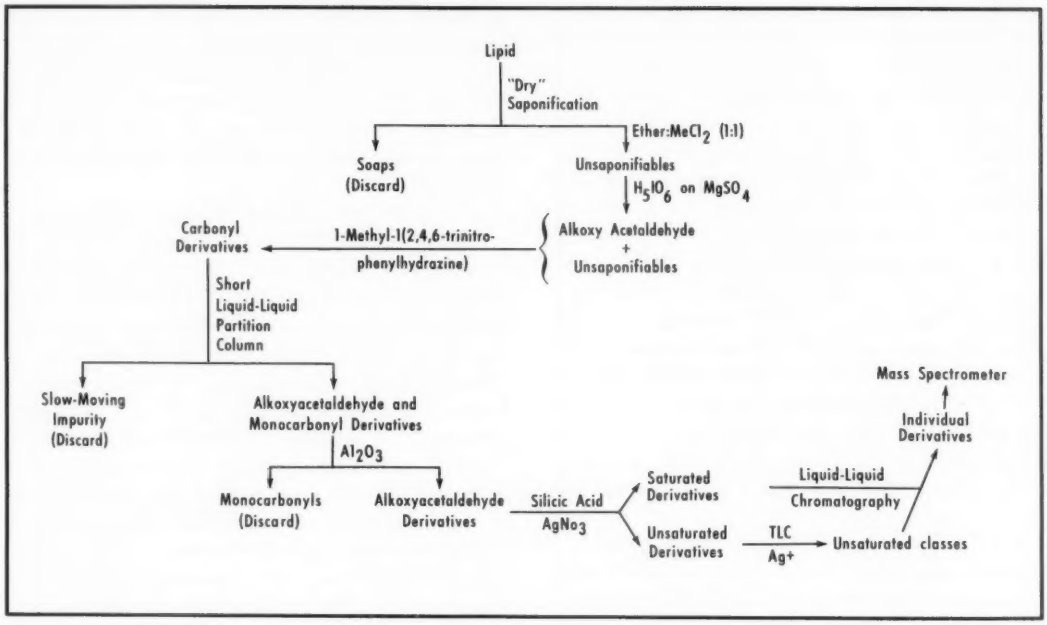

FIGURE 4—Schematic for the quantitative and qualitative determination of glyceryl ethers in lipids

consequently can be employed in practically any laboratory. It can also serve as a useful check on instrumental procedures.

Reaction columns are extensively used throughout the isolation and fractionation procedure, and the "solventless" saponification and periodic acid column techniques were virtually indispensable to the success of the overall method.

Some 70 glyceryl ethers were found in the unsaponifiable matter of milk fat. In an earlier study which used gas-liquid chromatography to resolve the glyceryl ether fraction, only three were found (*2*).

Milk fat was found to contain approximately 0.27 μmoles of glyceryl ethers per gram of which 70 percent are saturated. An homologous series of ethers from C_{10} through C_{18} was established in the saturated fraction, and about 55 unsaturated ethers were found. Colostrum, the first milk secreted after parturition, was found to be relatively high in glyceryl ethers, being some 4 to 6 fold more than those in normal milk.

A study of a number of plant oils (corn, olive, wheat germ, coconut, avocado, tung, castor, rice) indicated that these are completely devoid of glyceryl-1- alkyl ethers. This fact may be helpful in detecting the adulteration of plant oils with milk fat.

Determination of 1-Monoglycerides in Lipids

THE 1-monoglycerides, like other organic compounds containing adjacent hydroxyl groups, are oxidized readily by periodic acid. This reaction forms the basis for methods to determine 1-monoglycerides in lipids. Other structures occuring in lipids, however, can also be oxidized by periodic acid—thereby resulting in a false 1-monoglyceride value if the determination is made on the formaldehyde liberated in the oxidation. Also, it is not possible to determine the nature of the fatty acid in the monoglyceride without going through detailed chromatographic separations.

In a continuing effort to formulate new and improved procedures for analyzing lipids for their constituents, the method outlined in fig. 5 was developed. The method is simple to run, requires no elaborate equipment, and is applicable to very small concentrations of 1-monoglycerides. The identity of the fatty acid is readily determined by thin-layer

partition chromatography with the authentic 2,4-dinitrophenylhydrazones of the glycolaldehyde esters of the fatty acids (*17*).

Note that column reaction methods are extensively utilized throughout the procedure. The classical bisulfite reaction with aldehydes has been adapted for use with micro columns and is very effective in this form. No absorbents are used in the presence of triglycerides or partial glycerides which ordinarily could lead to artifact formation.

The quantitative aspects of the method have not been fully worked out at this writing.

Removal of 3β-Sterols

MILK fat is relatively rich in cholesterol and this fact practically precludes the determination of micro amounts of other hydroxylated moieties in the fat or in the unsaponifiable matter of the fat unless this sterol is first removed. The removal of cholesterol quantitatively from milk fat was rather simply done by impregnating digitonin onto Celite and passing the fat dissolved in hexane or benzene over this material packed into a column (*13*). Not only did the column quanitatively remove free cholesterol from the fat, but it did so without the use of an alcoholic solvent (the usual medium for cholesterol precipitation with digitonin) thereby eliminating an almost certain source of interference during the analysis of the fat for hydroxyl constituents.

Besides fulfilling the intended purpose, advantage has been taken of the digitonin column by utilizing it for the analysis of the sterols removed by it. Gas chromatography of the sterols removed from the digitonin column gives a pattern that can be used to gauge adulteration of milk fat with vegetable oil (*3*).

Saponification

IN order to examine the alcohols present in the unsaponifiable matter of lipids, a method was developed which could be used routinely with confidence for obtaining the unsaponifiable matter. It was essential that the use of alcoholic solvents be avoided since even traces of alcohol remaining in the unsaponifiable matter could undermine any attempt at quantitative analysis of hydroxy compounds. A saponification procedure was accordingly developed which employed no solvent in the actual saponification step and yet gave complete saponification (*18*). This was attained by grinding the fat or oil with aqueous potassium hydroxide in a mortar until saponification visibly ensued. Saponification was completed by heating the mortar for 20 minutes at 100° C. The soap is subsequently ground into a powder, or dispersed on Celite, or mixed with some anhydrous sodium sulfate and then extracted with benzene or methylene chloride to obtain the unsaponifiable matter.

The procedure is remarkably effective, relatively rapid, and besides avoiding the use of alcoholic solvents, it avoids the formation of the troublesome emulsions usually encountered in the standard saponification methods. Because of its simplicity, quantitative nature, and reproductibility, it became the forerunner of several other methods described in this manuscript.

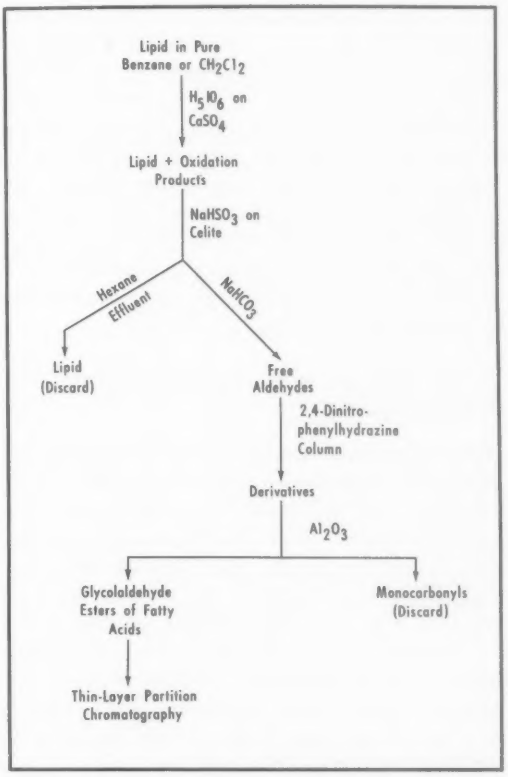

FIGURE 5—Isolation and characterization of 1-monoglycerides from lipids.

Chromic Acid Oxidation

THE position of a carbonyl group may be located via the Beckmann rearrangement of the oxime and subsequent hydrolysis of the amide following the rearrangement. The resulting fragments can usually be readily identified, allowing one to establish the position of the carbonyl group. In conjunction with an investigation on the hydroxylated constituents of milk fat, the location of the hydroxyl groups in the isolated alcohols was anticipated. Since many of the alcohols occurring in lipids are found in very small amounts, it was necessary to have on hand a procedure for the oxidation of the hydroxyl group to the keto group at the submicromole level and in high yield. A method was therefore developed to carry out the oxidation by using aqueous chromic acid impregnated on the surface of Celite (*23*).

In this procedure, which is carried out in micro columns, the alcohol dissolved in CCl_4 is passed over the column. The corresponding carbonyl compound emerges from the column and can be analyzed by gas or thin-layer chromatography, or derivatized. Quantitative oxidation of all secondary alcohols takes place rapidly. Thus, time-consuming extractions or evaporations are avoided, as is the use of glacial acetic acid—the most popular solvent for carrying out the oxidation in a homogeneous system. Unfortunately, the oxidation of unsaturated alcohols results in some side reaction products, and primary alcohols give relatively poor yields of the corresponding aldehydes.

Fractionation of Complex Mixtures on Micro Reaction Columns

AN insight into just how complex most natural products are has been gained through the use of gas-liquid and thin-layer chromatography. The multitude of both volatile and non-volatile constituents complicates analyses and, in many instances, necessitates some type of fractionation prior to chromatographic analysis. In our laboratory, an effort has been made to simplify complex mixtures by selectively removing classes quantitatively from the mixture under mild conditions and without causing unduly large dilution of the original solution. This is accomplished on relatively high capacity micro columns containing one of the reactants. The schematic of fig. 6 illustrates the fractionation of a mixture of fatty acids, amines, thiols and alcohols. A specific use of this scheme was to quantitatively determine both monohydric and dihydric alcohols present in a mixture containing amines and thiols (*22*). Both the amines and thiols interfere in the determination by directly reacting with the reagent (pyruvic acid chloride 2,6-dinitrophenylhydrazone) and the fatty acids interfere indirectly by combining with the reagent, making it inaccessible.

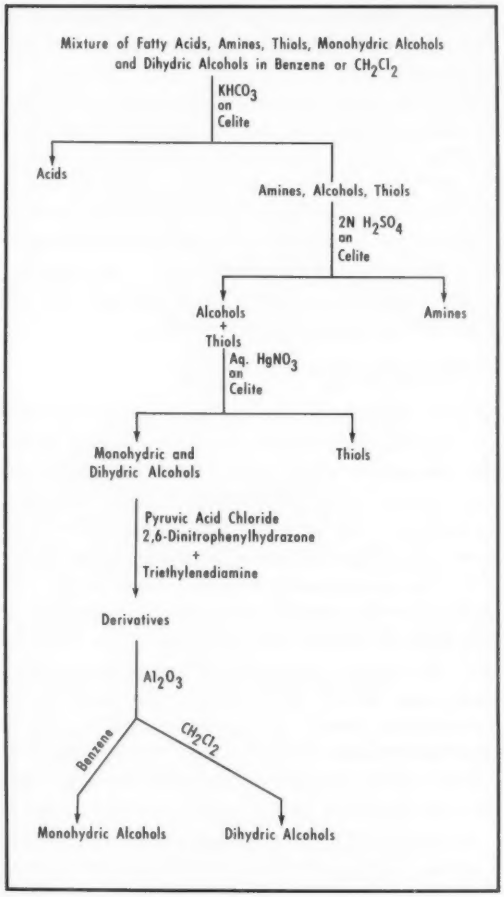

FIGURE 6—Fractionation of a mixture of fatty acids, amines, thiols, monohydric alcohols, and dihydric alcohols.

For the gas chromatography of volatiles, the selective removal of classes, besides simplifying the

chromatogram, enables one to simultaneously classify missing peaks. The same would be true for thin-layer chromatography of non-volatile components.

The efficacy of running reactions in a two-phase system on the surface of an inert support is again illustrated in the scheme, particularly in the use of bicarbonate as an extractant for fatty acids. Under ordinary extraction of fatty acids by aqueous base, that is, in a separatory funnel, bicarbonate would not be used, because it would fail to extract long-chain fatty acids. Stronger bases such as carbonate or hydroxide would be employed. The micro bicarbonate column, on the other hand, extracts all chain-length acids and does so quantitatively. Moreover, the use of stronger bases is avoided, virtually eliminating the danger of saponification of esters which would result in artifacts.

Incorporation of an acid-base indicator in the bicarbonate column permits one to follow the extraction visually, and also gives some indication of the magnitude of the fatty acid concentration in the sample. The mercurous nitrate column used for the extraction of the thiols is self-indicating, turning blue-grey as thiols react on it.

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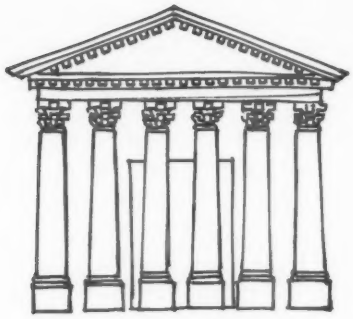
ERRATUM

In Dr. Willard J. Visek's article, "Urease: Its Significance in Birds and Mammals" (Vol. 8 No. 1), the author of one of the literature citations was inadvertently misquoted. The error occurred on page 21, first column, lines 7–9. The correct version should read:

"Cancer is a group of diseases found in all races and ages of man and in all other animal species . . . . Since 1937 cancer has been the second most frequent cause of death in the United States." (*3*)

Review regrets this error.



FORUM

WHAT IS AGRICULTURE?

THE classic definition of agriculture—as derived from the Latin roots *ager* (field) and *cultura* (cultivation)—is "the science and art of cultivating the soil, producing crops, and raising livestock." The word "farming" seems to be a commonly accepted synonym.

Such a definition is woefully inappropriate today—not so much because agriculture has changed, but because it encompasses a far broader scope of human activity than simply tilling the soil, tending a flock of chickens, or gathering in the traditional harvest.

True, the term "farming" does encompass the production processes listed in the dictionary definition. But such processes include managerial skills and the procurement and use of a wide variety of goods and services to a greater degree than most people realize. Off-farm capital, fertilizers, chemicals, machinery, and consulting services—all are an integral part of a modern agricultural enterprise. Moreover, farmers more often than not are involved in the affairs of numerous community and public activities associated with land use and the initial production process. Thus, many other people become associated by necessity with the initial production process, even though they and their organizations are physically separated from the rural scene.

A more definitive term than the word, farming, is the expression, "production agriculture," particularly as it is used in the context of the initial farm processes. Production agriculture, as a form of terminology, draws attention not only to the processes and the people involved with them, but also to all institutions associated in any way with the actual production of goods in commercial agriculture. Yet the term, production agriculture—by its very nature—implies certain limitations.

During World War II, the term, "agribusiness," was coined to more accurately describe the concept of modern agriculture. It includes the commercial and industrial components of all the foregoing items and their counterparts in the assemblying, processing, manufacturing, and synthesis of the following:

- New products derived wholly or partially from crops and livestock, with or without the addition of other products.
- A wide variety of substitute products.

Agribusiness also includes the handling and sale of raw food, feed, and fiber products—plus all the inputs of the private sector from point or origin to the point of final consumption. Much of what we term "agribusiness" was done on the farm when Webster's definition was written. Now, many of the functions of the agribusiness enterprise are performed off the farm.

But even the term "agribusiness" has limitations. Consider, for example, the production of forest trees in contrast to the production of fruit trees; a golf course turf in contrast to pasture grass; or a flower crop instead of a vegetable crop. Is one agricultural production and the other not? Or, if a fertilizer company sells its products to farmers, golf course operators, and urban homeowners, is the company "agricultural" only part of the time—when selling to farmers—and non-agricultural the rest of the time? Such a dichotomy seems highly illogical.

Today it is becoming more and more difficult to distinguish between production and marketing and other segments of the total production-to-consumer process. Many commercial firms are so organized that they encompass the total spectrum either by ownership of production, processing, and direct sales components or by ownership of some phases of operation and agreements and contracts with others. Such integration is occurring forward (toward the consumer) and backward (toward the farmer).

Another reason that the term "agribusiness" lacks definitive scope is that agriculture encompasses social implications for both public and private sectors. Conservation, wise resource use, environmental

quality, nutrition, ecology, and many other social concerns—all are now drawing heavily on the many-faceted resources of agriculture.

So how shall we define agriculture? Is it fair to regard it as a finite, measurable entity? We think not. Rather it is more of a field of emphasis. It is a focus for the efforts of many people: farmers, capitalists, manufacturers, university and government personnel and a host of other support people. Resources, knowledge, management skills, and other vital inputs are organized to improve productivity and profit, consumer service and enjoyment, convenience and economy. All of these elements—directed toward long-term public satisfaction—together they signify *agriculture.*

Mark T. Buchanan, Director at Large, Western Association of Agricultural Experiment Station Directors

G. F. MacLeod, Director, Liaison Study of Agricultural Education, University of California, Davis

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FOOD PRODUCT DEVELOPMENT RESEARCH (*Continued from page 12*)

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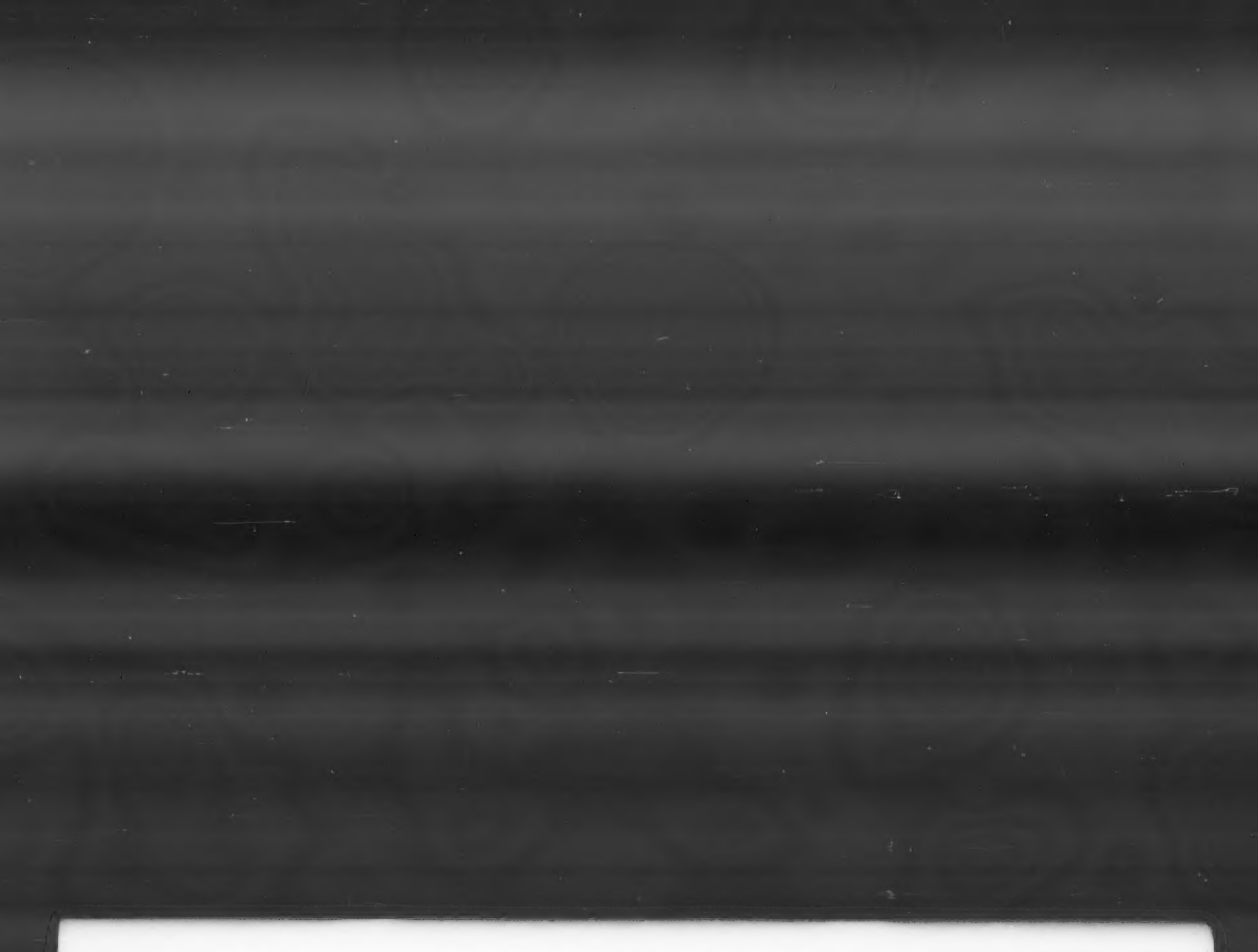
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